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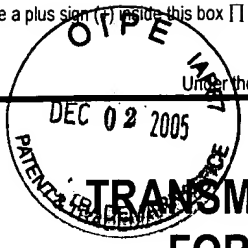

TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Application Number	09/981,547
Filing Date	OCTOBER 17, 2001
First Named Inventor	JIM WELLS
Group/Art Unit	1639
Examiner Name	EPPERSON, JON D.
Total Number of Pages in This Submission: 183	Attorney Docket Number: 39750-0002 DV1

ENCLOSURES *(check all that apply)*

- ☒ **FEE TRANSMITTAL FORM**
- ☐ Fee Attached
- ☐ Amendment / Response
- ☐ After Final
- ☐ Version With Markings Showing Changes
- ☐ Affidavits/declaration(s)
- ☐ Extension of Time Request
- ☐ Information Disclosure Statement
- ☐ Certified Copy of Priority Document(s)
- ☐ Response to Missing Parts/ Incomplete Application
- ☐ Response to Missing Parts under 37 CFR 1.52 or 1.53
- ☐ Copy of Notice
- ☐ Copy of an Assignment
- ☐ Drawing(s)
- ☐ Licensing-related Papers
- ☐ Petition Routing Slip (PTO/SB/69) and Accompanying Petition
- ☐ Petition to Convert to a Provisional Application
- ☐ Power of Attorney, by Assignee to Exclusion of Inventor Under 37 C.F.R. §3.71 With Revocation of Prior Powers
- ☐ Terminal Disclaimer
- ☐ Small Entity Statement
- ☐ Request for Refund
- ☐ After Allowance Communication to Group
- ☐ Appeal Communication to Board of Appeals and Interferences
- ☒ **APPEAL COMMUNICATION TO GROUP** ***(APPEAL NOTICE, BRIEF, REPLY BRIEF)***
- ☐ Proprietary Information
- ☐ Status Letter
- ☒ **ADDITIONAL ENCLOSURE(S)** ***(PLEASE IDENTIFY BELOW):***
- ☒ **EVIDENCE APPENDIX ITEMS 1-4;; and STAMPED RETURN POSTCARD**

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SIGNATURE OF APPLICANT, ATTORNEY OR AGENT

Firm *or* Individual name	HELLER EHRMAN LLP	GINGER R. DREGER (Reg. No. 33,055)	
	275 Middlefield Road, Menlo Park, California 94025	Telephone: (650) 324-7000	Facsimile: (650) 324-0638
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FEE TRANSMITTAL for FY 2005

Effective 10/1/2003. Patent fees are subject to annual revision.

☑ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) **500.00**

Complete if Known	
Application Number	09/981,547
Filing Date	OCTOBER 17, 2001
First Named Inventor	JIM WELLS
Examiner Name	EPPERSON, JON D.
Art Unit	1639
Attorney Docket No.	39750-0002 DV1

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FEE CALCULATION

1. BASIC FILING FEE

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1001	300	2001	150	Utility filing fee	
1002	200	2002	100	Design filing fee	
1003	200	2003	100	Plant filing fee	
1004	300	2004	150	Reissue filing fee	
1005	200	2005	100	Provisional filing fee	

SUBTOTAL (1) ($)

2. EXTRA CLAIM FEES FOR UTILITY AND REISSUE

		Extra Claims		Fee from below	Fee Paid
Total Claims		-20** =		X	=
Independent Claims		- 3** =		X	=
Multiple Dependent					=

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
1202	50	2202	25	Claims in excess of 20
1201	200	2201	100	Independent claims in excess of 3
1203	360	2203	180	Multiple dependent claim, if not paid
1204	200	2204	100	** Reissue independent claims over original patent
1205	50	2205	25	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) ($)

**or number previously paid, if greater; For Reissues, see above

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1051	130	2051	65	Surcharge - late filing fee or oath	
1052	50	2052	25	Surcharge - late provisional filing fee or cover sheet	
1053	130	1053	130	Non-English specification	
1812	2,520	1812	2,520	For filing a request for *ex parte* reexamination	
1804	920*	1804	920*	Requesting publication of SIR prior to Examiner action	
1805	1,840*	1805	1,840*	Requesting publication of SIR after Examiner action	
1251	120	2251	60	Extension for reply within first month	
1252	450	2252	225	Extension for reply within second month	
1253	1,020	2253	510	Extension for reply within third month	
1254	1,590	2254	795	Extension for reply within fourth month	
1255	2,160	2255	1,080	Extension for reply within fifth month	
1401	500	2401	250	Notice of Appeal	
1402	500	2402	250	Filing a brief in support of an appeal	500.00
1403	1,000	2403	500	Request for oral hearing	
1451	1,510	1451	1,510	Petition to institute a public use proceeding	
1452	500	2452	250	Petition to revive - unavoidable	
1453	1,500	2453	750	Petition to revive - unintentional	
1501	1,400	2501	700	Utility issue fee (or reissue)	
1502	800	2502	400	Design issue fee	
1503	1,100	2503	550	Plant issue fee	
1460	130	1460	130	Petitions to the Commissioner	
1807	50	1807	50	Processing fee under 37 CFR 1.17(q)	
1806	180	1806	180	Submission of Information Disclosure Stmt	
8021	40	8021	40	Recording each patent assignment per property (times number of properties)	
1809	790	2809	395	Filing a submission after final rejection (37 CFR 1.129(a))	
1810	790	2810	395	For each additional invention to be examined (37 CFR 1.129(b))	
1801	790	2801	395	Request for Continued Examination (RCE)	
1802	900	1802	900	Request for expedited examination of a design application	

Other fee (specify) _____

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SUBTOTAL (3) ($) **500.00**

SUBMITTED BY

(Complete *(if applicable)*)

Name (Print/Type)	GINGER R. DREGER	*Registration No. (Attorney/Agent)*	33,055	*Telephone*	(650) 324-7000
Signature				*Date*	DECEMBER 2, 2005

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Examiner: Epperson, Jon D.
Jim WELLS, et al.	Art Unit: 1639
Application Serial No. 09/981,547	Confirmation No: 8070
Filed: October 17, 2001	Attorney's Docket No. 39750-0002 DV1
For: **METHODS FOR RAPIDLY IDENTIFYING SMALL ORGANIC MOLECULE LIGANDS FOR BINDING TO BIOLOGICAL TARGET MOLECULES**	**Customer No. 25213**

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DATE MAILED: DECEMBER 2, 2005

ON APPEAL TO THE BOARD OF PATENT APPEALS AND INTERFERENCES
APPELLANTS' BRIEF

MAIL STOP APPEAL BRIEF - PATENTS
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Dear Sir:

On September 9, 2004, the Examiner finally rejected Claims 58, 59, 61, 65, 81-89, 93, 95 and 96 of this application. The rejection of the same claims was maintained following the filing of a Request for Continued Examination, in a non-final Office Action mailed on May 25, 2005. On August 25, 2005, Appellants filed a Notice of Appeal and a Pre-Appeal Brief Request for Review. According to the Notice of Panel Decision for Pre-Appeal Brief Review mailed on November 2, 2005, the application remains under appeal, and an Appeal Brief is due in accordance with 37 C.F.R. §41.37.

Appellants hereby appeal to the Board of Patent Appeals and Interferences from the rejection of Claims 58, 59, 61, 65, 81-89, 93, 95 and 96.

The following constitutes Appellants' Brief on Appeal.

1. REAL PARTY IN INTEREST

The real party in interest is Sunesis Pharmaceuticals, Inc., South San Francisco, California, by an assignment of parent application U.S. Patent Application Serial No. 09/105,372 (now U.S. Patent No. 6,335,155) recorded on August 31, 1998, at Reel: 009415 and Frame: 0304.

2. RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences.

3. STATUS OF CLAIMS

Claims 58, 59, 61-66 and 81-96 are in this application.

Claims 62-64, 66, 90-92 and 94 are withdrawn from consideration.

Claims 58, 59, 61, 65, 81-89, 93, 95 and 96 stand rejected and Appellants appeal the rejection of these claims.

A copy of the rejected claims involved in the present Appeal is provided in the Claims Appendix.

4. STATUS OF AMENDMENTS

There were no amendments to the claims submitted after final rejection. All previous amendments to the claims have been entered.

5. SUMMARY OF CLAIMED SUBJECT MATTER

The invention is directed to a new strategy, called "tethering," for site-directed ligand discovery.

As claimed in Claim 58, the invention is a method for identifying a non-oligomeric organic compounds that has the greatest relative affinity for the target protein from a library of non-oligomeric organic compounds, less than 2000 daltons in size, that are capable of binding covalently to a chemically reactive group on the target protein to form a target protein-ligand conjugate. The target protein and the library are contacted with each other in a mixture, and once a target protein-compound conjugate is formed, the mixture is analyzed by mass spectrometry.

Using mass spectrometry, (1) the target protein-compound conjugate is detected, (2) the identity of the non-oligomeric organic compound present in the conjugate is determined, and (3) the compound is identified as having the greatest relative affinity for the target protein from the compounds present in the library analyzed. Thus, the method results in the identification of a novel ligand for the target protein.

A library of non-oligomeric organic compounds less than 2000 daltons in size is disclosed, for example, on page 2, lines 1-5, in the passage bridging pages 4 and 5, and in the passage bridging pages 16 and 17 of the specification. Target proteins are disclosed at least on page 8, line 14 - page 10, line 12. Chemically reactive groups on target proteins and on members of the library of non-oligomeric organic compounds of less than 2000 daltons in size, and methods for introducing such reactive groups into target proteins are described, for example, at page 6, lines 10-31; page 9, line 13 - page 13, line 8; page 13, line 30 - page 14, line 9; and page 14, line 24 - page 15, line 14 of the specification. Screening a library with a target protein carrying a reactive group is described, for example, at page 15, line 15 - page 20, line 14; and page 17, line 24 - page 20, line 14. Mass spectrometry analysis is discussed at least at page 5, lines 18-23, and page 21, lines 17-25. Specific reference to a compound having the greatest relative affinity for the target protein from the compounds analyzed is at page 15, line 19 - page 16, line 3. Further support for this method is in the EXPERIMENTAL section at pages 24-28 of the specification.

Claim 59 recites that the ligand is less than 1500 daltons in size, and is supported at page 16, line 27 of the specification.

Claim 61 recites that the ligand is less than 750 daltons in size, and is supported at page 16, line 27 of the specification.

Claim 65 recites that the target protein is a TNF receptor. This claim is supported in the passage bridging pages 8 and 9.

Claim 81 recites that the chemically reactive group on the target protein is an -SH group, a protected -SH group or an activated -SH group. Claim 82 adds that the -SH group, protected -SH group or activated -SH group is part of a cysteine residue of the target protein. These claims

are supported at least at page 6, lines 10-21; and page 10, line 12 - page 13, line 8.

Claims 83-85 recite that the library comprises at least two, at least 25, and at least 100 members, respectively. These claims are supported in the paragraph bridging pages 18 and 19 of the specification.

Claim 86 is drawn to a competition assay, in which a target protein, a reducing agent, and at least two compounds that are less than 2000 daltons in size and are capable of forming a disulfide bond with the target protein are contacted in a mixture, the mixture is analyzed by mass spectrometry, and the most abundant target protein-compound conjugate formed is detected. Claim 87 states that the identity of the compounds that is disulfide bonded to the target protein in the most abundant target protein-compound conjugate is determined. Support for these claims is as for Claim 58. In addition, support for the "most abundant" target protein-ligand conjugate is at page 15, line 19 - page 16, line 3.

Claims 88 and 89 recite that the compounds are less than 1500 and 750 daltons, respectively, in size, and are supported at page 16, line 27 of the specification.

Claim 93 recites that the target protein is a TNF receptor, and is supported in the passage bridging pages 8 and 9.

Claims 95 and 96 state that the mixture is contacted with at least 25 and at least one hundred compounds, respectively. Support for these claims is in the paragraph bridging pages 18 and 19 of the specification.

6. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

Whether the rejection of Claims 58, 59, 61, 65, 81-89, 93, 95, and 96 under 35 U.S.C. § 103 over Kim *et al.* (WO 98/11436) and Siuzdak (Siuzdak, G., Mass Spectrometry for Biotechnology, New York, Academic Press, 1996, pp. 119-126) and Jindal *et al.* (WO 97/01755) is in error and should be withdrawn.

7. ARGUMENT

I. *History of the Rejections*

The combination of Kim *et al.* and Siuzdak was first cited in support of an obviousness

rejection in an Office Action mailed on December 29, 2003. The rejection was repeated in a final Office Action mailed on September 9, 2004 and maintained in an Advisory Action mailed on December 20, 2004.

Kim *et al.* was cited for describing a method of detecting a ligand that binds to a target molecule. The ligand and the target molecule each contains, as obtained or modified, a member of a binding pair, to permit covalent linkage or tethering of the ligand when bound to the target molecule. In one embodiment, the binding pair consists of sulfhydryl groups. The Examiner cited Siuzdak for its teaching of electrospray mass spectrometry, quoting the statement from the publication that this technique "demonstrated its potential in the analysis of non-covalent interactions between an antibody and a hapten, and for observing covalent protein-bound intermediates in an antibody-catalyzed reaction." (See page 9 of the Office Action of December 29, 2003; page 8 of the Office Action of September 9, 2004; and page 8 of the Advisory Action of December 20, 2004). In simple terms, the rejection is based on the premise that the Kim *et al.* reference teaches all elements of independent Claims 58 and 86, with the exception of using mass spectrometry to detect the target protein-compound conjugate, which, in turn, is said to be disclosed in Siuzdak.

Thus, in analyzing independent Claims 58 and 86, the Examiner notes that *"Kim et al. is deficient in that it does not specifically teach the use of mass spectrometry,"* but adds that *"the Siuzdak (see entire document) teaches the use of electrospray mass spectrometry to study both 'non-covalent' and 'covalent' antibody-antigen interactions including fragmentation techniques like MS^2 and MS^3."* (See page 8 of the Office Action of December 29, 2003; page 8 of the Final Office Action of September 9, 2004; and page 7 of the Advisory Action of December 20, 2004).

In response, Appellants argued that the references were not properly combined, and even if the combination had been proper, it would still not have made obvious the claimed invention.

In addition to scientific and legal arguments advanced in the responses filed to these Office Actions, with a Preliminary Communication dated February 28, 2005, filed concurrently with the filing of a Request for Continued Examination (RCE), Appellants submitted a Declaration of Gary Siuzdak, Ph.D., the sole author of the Siuzdak reference. According to the

paragraphs 3 and 4 of the Declaration, Dr. Siuzdak disagrees with the Examiner's conclusion that his quoted statement that "[e]lectrospray ionization mass spectrometry has also demonstrated its potential . . . for observing covalent protein-bound intermediates in an antibody-catalyzed reaction" would have motivated one skilled in the art to identify a novel ligand by the mass spectrometry detection of a covalently bound protein-ligand conjugate in a mixture. In paragraph 6, Dr. Siuzdak explains that *"[w]hile electrospray ionization mass spectrometry is well suited to study enzymatic mechanisms where all of the participants are known, its use to analyze mixtures of unknown components is limited."* One reason for this is that *"heterogeneous compounds can produce complicated spectra that can be difficult or impossible to interpret."* Another obstacle is that *"heterogeneous mixtures tend to reduce the sensitivity of electrospray ionization mass spectrometry."* Dr. Siuzdak adds that *"these obstacles are shared by other technique of mass spectrometry."* In view of this explanation, in paragraph 7 Dr. Siuzdak states: *"I do not believe that a person skilled in the art would have assumed that mass spectrometry techniques to study enzymatic mechanisms would have been applicable to identify novel ligands by the mass spectrometry analysis of a mixture of unknown chemical entities, detecting a covalently bound protein-ligand conjugate from among the chemical entities present in the mixture, and determining the identity of the ligand present in the conjugate detected."*

In an Office Action mailed on May 25, 2005, the Examiner maintained the rejection of all claims under examination under 35 U.S.C. §103, and, in support of the rejection, additionally cited Jidal *et al.*, stating that the teaching missing from Kim *et al.* is supplied by "the combined references of Siuzdak and Jindal *et al.*" (Page 7) Jindal *et al.* was combined with Kim *et al.* as allegedly showing that mass spectrometry can be applied to combinatorial libraries of targets and/or ligands for screening purposes. (Page 8, lines 2-9 of the Office Action of May 25, 2005, which erroneously refers to the combination of Siuzdak and Kim *et al.*, but clearly means Jindal *et al.* and Kim *et al.*). From this, the Examiner concludes that *"[I]t would have been obvious to one skilled in the art at the time the invention was made to 'identify' target/ligand interactions using the 'affinity enhancing' techniques as taught by Kim et al. with mass spectrometry as taught by the combined references of Siuzdak and Jindal et al., because Jindal et al., for*

example, explicitly state that mass spectrometry can be applied to the study of target/ligand interactions including the use of combinatorial libraries." (Page 8, last paragraph of the Office Action of May 25, 2005).

The Examiner found the Siuzdak Declaration not persuasive for the following reasons:

(1) *"Although Dr. Gary Siuzdak is unquestionably an expert in the field of mass spectrometry, his position in this particular case does not seem to be supported by the art."* (Page 12, last paragraph of the Office Action of May 25, 2005).

(2) In addressing Dr. Siuzdak's conclusion that a person skilled in the art at the time the present invention was made would not have assumed that the mass spectrometry techniques used to study enzymatic mechanisms would have been applicable to identify novel ligands by mass spectrometry analysis in a mixture of unknown chemical entities, detecting the covalently bound protein-ligand conjugate from among the chemical entities present in the mixture, and determining the identity of the ligand present in the conjugate detected, the Examiner cited Jindal *et al.*, as allegedly showing that *"mass spectrometry was 'routinely' applied to 'unknown' targets and/or ligands including 'complicated' mixtures like combinatorial libraries."* (Paragraph bridging pages 12 and 13 of the Office Action of May 25, 2005).

(3) Finally, the Examiner added that *"the Siuzdak reference is only one of potentially hundreds if not thousands of references"* that support his position. (Page 13 of the Office Action of May 25, 2005.) The Examiner further referred to the Advisory Action of December 20, 2004, presumably to point out the documents cited under the heading "References Illustrative of the State of the Prior Art," although this is not entirely clear, since these references have never been applied against the rejected claims. Thus, the Examiner has not cited any of the *"potentially hundreds if not thousands of references"* and the rejection is based solely on the combination of Kim *et al.* with Siuzdak and Jindal *et al.*

On August 25, 2005, Appellants filed a Pre-Appeal Brief Request for Review along with the filing of a Notice of Appeal. According to a Notice of Panel Decision from Pre-Appeal Brief Review dated November 2, 2005, the "application remains under appeal because there is at least one actual issue for appeal."

II. *Obviousness - legal standard*

A claimed invention is unpatentable for obviousness if the differences between it and the prior art "are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art." 35 U.S.C. §103(a); Graham v. John Deere Co., 383 U.S. 1, 14, 148 USPQ 459, 465 (1966). Obviousness is a legal question based on underlying factual determinations including: (1) the scope and content of the prior art; (2) the level of ordinary skill in the prior art; (3) the differences between the claimed invention and the prior art; and (4) objective evidence of nonobviousness. Graham, 383 U.S. at 17-18, 148 USPQ at 467.

"[I]t is fundamental that rejections under 35 U.S.C. §103 must be based on evidence comprehended by the language of that section." In re Grasselli, 713 F.2d 731, 739, 218 USPQ 769, 775 (Fed. Cir. 1983).

When obviousness is based on the teachings of multiple prior art references, the U.S. Court of Appeals for the Federal Circuit has held that

> [t]he PTO has the burden under section 103 to establish a *prima facie* case of obviousness . . . It can satisfy this burden only by showing some objective teaching in the prior art or that knowledge generally available to one of ordinary skill in the art would lead that individual to combine the relevant teachings of the references. In re Fine, 837 F.2d 1071, 1074 (Fed. Cir. 1988).

The case law is also clear that the motivation to support a combination of references in a Section 103 rejection must withstand scrutiny. In In re Rouffet, 149 F.3d 1350; 47 USPQ2d 1453 (Fed. Cir. 1998) , the C.A.F.C. reaffirmed that a suggestion to combine known elements present in various pieces of prior art is critical for establishing a *prima facie* case of obviousness. The C.A.F.C. observed that:

> "[V]irtually all [inventions] are combinations of old elements." Environmental Designs, Ltd. V. Union Oil Co., 713 F.2d 693, 698, 218 U.S.P.Q. 865, 870 (Fed. Cir. 1983); see also Richdel, Inc. v. Sunspool Corp., 714 F.2s 1573, 1579-80, 219 U.S.P.Q. 8, 12 (Fed. Cir. 1983) ("Most, if not all, inventions are combinations and mostly of old elements."). Therefore, an examiner may often find every element of a claimed invention in the prior art. If identification of

> each claimed element in the prior art were sufficient to negate patentability, very few patents would ever issue. Furthermore, rejecting patents solely by finding prior art corollaries for the claimed elements would permit an examiner to use the claimed invention itself as a blueprint for piecing together elements in the prior art to defeat the patentability of the claimed invention. Such approach would be "an illogical and inappropriate process by which to determine patentability." Sensonics, Inc. v. Aerosonic Corp., 81 F.3d 1566, 1570, 38 U.S.P.Q.2d 1551, 1554 (Fed. Cir. 1996). In re Rouffet, 149 F.3d at 1357 47 U.S.P.Q.2d at 1457

The requirement that an examiner must show a suggestion to combine references cited in support of an obviousness rejection is a critical safeguard against hindsight reconstruction of an invention. The motivation to modify a reference can come from: (1) the nature of the problem to be solved, (2) the teachings of the prior art itself, or (3) the knowledge of persons of ordinary skill in the art. In re Rouffet, 149 F.3d at 1358; 47 U.S.P.Q.2d at 1458.

> "Combining prior art references without evidence of such a suggestion, teaching or motivation simply takes the inventor's disclosure as a blueprint for piecing together the prior art to defeat patentability - the essence of hindsight." In re Dembiczak, 175 F.3d 994, 999 (Fed. Cir. 1999).

It has been well established that

> "[t]he Board cannot simply reach conclusions based on its own understanding or experience - or on its assessment of what would be basic knowledge or common sense. Rather, the Board must point to some concrete evidence in the record in support of this finding." In re Zurko, 258 F.3d 1379, 1386, 59 USPQ2d 1693, 1697 (Fed. Cir. 2001).

Common knowledge or common sense may only be applied to analysis of the evidence, rather than be a substitute for evidence. In re Lee, 277 F.3d 1338, 61 USPQ2d 1430 (Fed. Cir. 2002).

III. ***The rejection of claims 58, 59, 61, 81-89, 93, 95, and 96 is legally and scientifically improper and should be withdrawn.***

1. *The Examiner's dismissal of the Siuzdak Declaration is improper*

It is well established that Office personnel must accept an opinion from a qualified expert based on relevant facts. It is improper to disregard such opinion solely because the Examiner disagrees with its conclusions. This is exactly what happened in the present case. While the Examiner has acknowledged that declarant, and the sole author of the cited Siuzdak reference, is unquestionably an expert in the field, Dr. Siuzdak's conclusions were dismissed, since they were allegedly contradicted by Jindal *et al.,* and by "potentially hundreds if not thousands of references," none of which were cited or applied against the claims pending. As it will be shown, Jindal *et al.* is yet again from another technical field, addressing a materially different problem from the problem addressed by the present invention, and thus does not contradict the conclusions of the Siuzdak Declaration. Indeed, it would be surprising if the Examiner could cite even one reference (let alone hundreds or thousands of references) that could validly contradict a renown scientist's explanation and conclusions drawn from his own work.

2. *There is no motivation to combine Kim et al. and Siuzdak and Jindal et al.*

The requirement that an examiner must show a suggestion to combine references in support from an obviousness rejection is a critical safeguard against hindsight reconstruction of an invention. As discussed above, the motivation to modify a reference can come from: (1) the nature of the problem to be solved, (2) the teachings of the prior art itself, or (3) the knowledge of persons of ordinary skill in the art. In re Rouffet, 149 F.3d at 1358; 47 U.S.P.Q.2d at 1458.

a. The motivation to combine Kim *et al.* and Siuzdak and Jindal *et al.* does not derive from the nature of the problem to be solved.

In the case of the present invention, the relevant problem to be solved is: (1) the detection, in a mixture of target protein-compound conjugates, the most abundant conjugate, and the determination of the identity of the compound present in the conjugate, which will be the compound having the greatest relative affinity for the target protein of the compounds present in the mixture assayed (Claim 58); or (2) the detection of the most abundant protein-compound

conjugate formed in a mixture containing the target protein, at least two compounds that are capable of forming a conjugate with the target protein through disulfide bond formation, and a reducing agent (Claim 86). The Siuzdak Declaration clearly establishes that mass spectrometry would not have been a method of choice for either case. The citation of Jindal *et al.* does not change this conclusion, since Jindal *et al.* does not contradict the conclusion of the Siuzdak Declaration. Jindal *et al.* disclose a complex chromatography-based screening method for identifying a ligand for a target of interest in a peptide library. The mixture screened contained three types of peptides: those that i) have no affinity to any protein; (ii) bind to a large number of proteins; and (iii) show affinity to a specific target protein. The screening system of Jindal *et al.* differentiates among these various peptides using a series of chromatographic steps, each based on a different physico-chemical characteristic, ultimately leading to the separation of one or more ligands for the target protein, which are then identified by any method known in the art suitable for detection, including mass spectrometry and subsequent peptide sequencing. This is in contrast with to the present invention, which does not involve such separation steps, and where a complex mixture of target protein-ligand conjugates and optionally ligand candidates is analyzed by mass spectrometry, allowing the determination of the identity of a particular ligand from among the conjugates and ligand candidates present, which typically tend to have very similar molecular weights. Since neither Siuzdak nor Jindal *et al.* address the problem addressed by the present invention, and Siuzdak and Jindal *et al.* each addresses a different and distinct problem, the motivation to combine these references with Kim *et al.* in an attempt to arrive at the claimed invention does not derive from the nature of the problem to be solved.

b. The motivation to combine Kim *et al.* and Siuzdak and Jindal *et al.* does not derive from the teachings of the cited prior art

The only detection method specifically disclosed in Kim *et al.* is detection using antibodies (page 21, second sentence of first full paragraph). Mass spectrometry is nowhere mentioned or suggested. Siuzdak teaches the use of electrospray ionization mass spectrometry for studying noncovalent binding between an antibody and its antigen, both known entities. Dr. Siuzdak, author of the cited chapter, unambiguously states in his opinion that from this

teaching one would not have assumed that a similar approach would work *"to identify novel ligands by mass spectrometry analysis of a mixture of unknown chemical entities, detecting a covalently bound protein-ligand conjugate from among the chemical entities present in the mixture, and determining the identity of the ligand present in the conjugate detected."* Although Jindal *et al.* uses mass spectrometry, it uses it in a context that differs significantly both from the methods of the present application and from the much simpler methods disclosed in the Siuzdak reference. Accordingly, there is nothing in the respective teachings of the cited references that would provide a motivation for their combination.

c. The motivation to combine Kim *et al.* and Siuzdak and Jindal *et al.* does not derive from the knowledge of persons of ordinary skill in the art.

Dr. Siuzdak, an unquestionable person skilled in the pertinent art, cannot see the motivation to combine Kim *et al.* and Siuzdak. Accordingly, the Siuzdak Declaration creates a strong presumption that such motivation does not exist. The Examiner's attempt to discredit Dr. Siuzdak's statements by citing a reference (Jindal *et al.*) using mass spectrometry is a method that significantly differs from the method of the present invention is not sufficient to overcome this presumption. Thus, the conclusion remains that one of ordinary skill, based on general knowledge in the art at the time the present invention was made, would not have been motivated to combine Kim *et al.* and Siuzdak and Jidal *et al.*

3. *The cited references, even if they could be properly combined, do not make obvious the claimed invention*

The Siuzdak Declaration makes it clear that, based on the combined teaching of Kim *et al.*, which discusses a tethering method but has no mention of mass spectrometry, and Siuzdak, discussing the use of electrospray mass spectrometry to study interactions where all participants are known, one skilled in the art would not have a reasonable expectation that mass spectrometry can be used to identify novel ligands by analysis of a mixture of unknown chemical entitites, detecting a covalently bound protein-ligand conjugate from among the chemical entities present in the mixture, and determining the identity of the ligand present in the conjugate detected. As Dr. Siuzdak explains in paragraph 6 of his Declaration *"[w]hile electrospray ionization mass*

spectrometry is well suited to study enzymatic mechanisms where all of the participants are known, its use to analyze mixtures of unknown components is limited." One reason for this is that *"heterogeneous compounds can produce complicated spectra that can be difficult or impossible to interpret."* Another obstacle is that *"heterogeneous mixtures tend to reduce the sensitivity of electrospray ionization mass spectrometry."* Dr. Siuzdak adds that *"these obstacles are shared by other technique of mass spectrometry."* In view of this explanation, in paragraph 7 Dr. Siuzdak states: *"I do not believe that a person skilled in the art would have assumed that mass spectrometry techniques to study enzymatic mechanisms would have been applicable to identify novel ligands by the mass spectrometry analysis of a mixture of unknown chemical entities, detecting a covalently bound protein-ligand conjugate from among the chemical entities present in the mixture, and determining the identity of the ligand present in the conjugate detected."*

The Examiner citation of Jindal *et al.* does not effectively rebut this conclusion.

The Examiner refers to the following statements from Jindal *et al.*:

(1) *"the present invention is directed to rapid, efficient, and automated . . . methods . . . for screening libraries to select . . . a candidate ligand. . . for a preselected target molecule." "the present invention . . . overcomes the disadvantages of the method known in the art."* (Jindal *et al,* page 5, paragraphs 1-2, emphasis added by Examiner.)

(2) *"Screening methods known in the art are not entirely satisfactory . . . existing systems are unable selectively to screen a library while simultaneously determining the affinity of selected ligands for the target . . .* [another] *major hurdle* [that has overcome by the present invention] . . . *is effective chemical characterization of ligands identified in these processes. . . . A major focus . . . is to enable the collection of enough of or enough information about a ligand of inerest so as to permit determination of its structure* [i.e. using mass spectroscopy]." (Jindal *et al.,* page 3, lines 11-24, the Examiner's own additions shown in brackets).

The Examiner reads the foregoing statements to mean that *"a person skilled in the art would have been motivated to use mass spectrometry . . . because Jindal et al., for example, state that their technique improves upon the prior art by increasing the speed by which the*

target/ligand interactions can be screened, facilitating the use of automation, increasing the sensitivity of the method, and provide enough information about the ligand to facilitate its molecular 'identification' thus preventing the need for further characterization by some other analytical technique." (Paragraph bridging pages 8 and 9 of Office Action of May 25, 2005.)

The Examiner further cites from Jindal *et al.*:

(3) . *The integrated coupling of various dimensions such as . . . electrospray ionization mass spectrometry in an automated multi-dimensional system should permit a highly sensitive and highly selective approach to decoding complex mixtures* [*i.e.,* mass spectroscopy is the choice for libraries]." (Jindal *et al.*, page 26, lines 12-15, the Examiner's own addition/comment shown in brackets).

The Examiner characterizes the cited statement to mean that *"Jindal et al. explicitly state that mass spectrometry is the method of choice for studying libraries."* (Page 9 of Office Action of May 25, 2005).

Starting with cited statement (1), it is merely an assertion, commonly present in patent applications, asserting that the invention disclosed and claimed in Jindal *et al.* represents certain advances over the prior art. Since the cited paragraph has no reference to mass spectrometry detection, and does not state, suggest or imply that mass spectrometry detection would be responsible for or even part of the reasons for the stated advances, it has no bearing on the patentability of the invention claimed in the present application.

If possible, statement (2) has even less bearing on the issue of obviousness of the invention claimed in the present application. The selectively cited statements are from the "Background" section of the Jindal *et al.* PCT publication, and in full read as follows:

"Screening methods known in the art thus are not entirely satisfactory. ***Prior methods for detecting or identifying ligands which bind to a target of interest often fail to provide ligands of sufficiently high affinity to be useful, and additionally suffer from the loss of sample, the need for large amounts of ligands, and the need to vary loading, binding, or elution conditions to obtain useful results.*** ***Additionally,*** *existing systems are unable selectively to screen a library while simultaneously determining the affinity of selected ligand(s) for the target* ***under relevant conditions.***

A major hurdle ***in the exploitation of current screening techniques of the type described above*** *is effective chemical characterization of ligands identified in these processes.* ***Chemical characterization, e.g. determining the sequence of an identified biopolymer, is at best time-consuming and complex.*** *A major focus* ***of prior art screening techniques*** *is to enable the collection of enough of or enough information about a ligand of interest as to permit determination of its structure* ***and to enable synthesis of larger amounts for testing and further empirical structural refinement."*** (Jindal *et al.*, page 3, lines 11-24, where the statement omitted by the Examiner are highlighted.)

The Examiner's selective citation is apparent from the extensive bolded statements, which have been omitted by the Examiner. The Examiner's substitution of his own thoughts in the middle of citations, such as "[that has overcome by the present invention]" and "[*i.e.* using mass spectroscopy]" is particularly troubling. As noted above, the cited paragraphs are from the Background section of the Jindal *et al.* PCT publication. While one can assume that these statements are present to emphasize the disadvantages of the prior art methods, and lead up to explaining the advanced represented by the Jindal *et al.* invention over such prior art methods, there is nothing in the cited statements that would indicate show which, if any, of the alleged drawbacks of prior art methods have overcome by Jindal *et al.,* and what part, if any, mass spectrometry has played in the alleged advances represented by the Jindal *et al.* invention. Accordingly, the insertion of the Examiner's bracketed statements lacks any support and is entirely unjustified, showing the Examiner's bias in reading this reference.

Turning to citation (3), the full sentence from page 26, lines 12-15 of Jindal *et al.*, where the parts omitted by the Examiner are bolded, reads as follows:

"The integrated coupling of various dimensions such as **micro column affinity chromatography with capillary reverse phase HPLC**/electrospray ionization mass spectrometry in an automated multi-dimensional system should permit a highly sensitive and highly selective approach to decoding complex mixtures."

To read this sentence as an explicit statement "that mass spectroscopy is the method of choice for studying libraries" is a clear misrepresentation. What the sentence says, when cited in full, is that coupling micro column affinity chromatography with capillary reverse phase HPLC/electrospray ionization mass spectrometry "should" permit a highly sensitive and selected approach to decoding complex mixtures. There is nothing in the statement that would indicate

that electrospray ionization mass spectrometry alone, without being combined with micro column affinity chromatography would provide the stated benefits. Indeed, the entire disclosure of Jindal *et al.,* including Figures 1 and 2, clearly shows that Jindal *et al.* always use mass spectrometry as part of a complex analytical system, following a variety of chromatographic separation steps.

Furthermore, there is nothing in this statement that could be reasonably read to mean that "*mass spectrometry is the method of choice for studying libraries*" as the Examiner states, although mass spectrometry methods are described as methods of detection elsewhere (*e.g.* page 4, lines 9-22).

In addition, Jindal *et al.* do not disclose mass spectrometry for the analysis of target protein-compound conjugates, as disclosed and claimed in the present application. As illustrated by the examples of Jindal *et al.,* the use of mass spectrometry is taught to detect peptides following various chromatographic (*e.g.* size exclusion, reverse phase chromatography) separation steps, which peptides can be subsequently recovered, if needed. (See, *e.g.* page 42, lines 4-11). In one embodiment, "a selected ligand" is introduced in a mass spectrometer "for determination of the charge to mass ration of the ligand." (Page 44, lines 10-13.) In another embodiment, after several chromatographic separation steps, a sample from the last exit stream is directed to a mass spectrometer, and the information is transmitted to an output display. (Passage bridging pages 44 and 45 of Jindal *et al.*) Based on the numbering, the latter embodiment is illustrated in Figure 4, where a mass spectrum is included to show the mass/charge ratios for the components of the sample analyzed. Mass spectra are also shown in Figures 7A-D.

Since Jindal *et al.* do not teach or suggest the use of mass spectrometry to analyze a mixture comprising target protein-compound conjugates, to detect the most abundant target protein-compound conjugate (Claims 58 and 86), and to determine the identity of a non-oligomeric organic compound present in such conjugate (Claim 58), this reference does not contradict the conclusion of the Siuzdak Declaration that as person skilled in the art would not have assumed "*that mass spectrometry techniques to study enzymatic mechanisms would have been applicable to identify novel ligands by the mass spectrometry analysis of a mixture of*

unknown chemical entities, detecting a covalently bound protein-ligand conjugate from among the chemical entities present in the mixture, and determining the identity of the ligand present in the conjugate detected." (Emphasis added.)

In conclusion, the out of context quotes from Jindal *et al.*, generously supplemented by the Examiner's own thoughts, do not support the rejection, rather are an illustration of the Examiner's attempt of hindsight reconstruction of the claimed invention, based on the Examiner's own notion of common knowledge in the art, and despite an expert's Declaration to the contrary.

From the foregoing analysis it should be clear that the Examiner in fact bases the present rejection on the unsupported and preconceived notion that the use of mass spectrometry for the purpose used in the present application was common knowledge and common sense at the priority date of the present application. This is particularly clear in view of the misinterpretation of Jindal *et al.*, which does not support the conclusions for which it is cited, and the Examiner's references to potential hundreds or thousands of other references that could have been, but have not been, cited.

Since the Examiner clearly has substituted his own opinion for actual relevant evidence, disregarding the explanation of a person skilled in the art of the meaning and implications of his own publication, the Board should conclude that the Examiner has not set forth a *prima facie* case of obviousness.

The Federal Circuit has clearly established that

> *"[T]he Board cannot simply reach conclusions based on its own understanding and experience - or on its assessment of what would be basic knowledge or common sense. Rather, the Board must point to some concrete evidence in the record in support of these findings."* In re Zurko, 258 F.3d 1379, 1386, 59 USPQ2d 1693, 1697 (Fed. Cir. 2001).

As the court determined in In re Lee, common knowledge and common sense may only be applied to the analysis of evidence, rather be a substitute for evidence. 277 F.3d 1338, 1345, 61 USPQ2d 1430, 1435 (Fed. Cir. 2002).

CONCLUSION

For the reasons given above, Appellants respectfully submit that Claims 58, 59, 61, 65, 81-89, 93, 95, and 96 are not made obvious under 35 U.S.C. §103 by the cited combination of references. Accordingly, the reversal of the present rejections is respectfully requested.

Respectfully submitted,

Date: December 2, 2005

By: _____
Ginger R. Dreger (Reg. No. 33,055)

HELLER EHRMAN LLP
275 Middlefield Road
Menlo Park, California 94025-3506
Telephone: (650) 324-7000
Facsimile: (650) 324-0638

8. CLAIMS APPENDIX

Claims on Appeal

58. A method of identifying a non-oligomeric organic compound that has the greatest relative affinity for a target protein comprising:

(a) contacting in a mixture a target protein with a library of non-oligomeric organic compounds, less than 2000 daltons in size, that are each capable of binding covalently to a chemically reactive group on the target protein, thereby forming a target protein-compound conjugate;

(b) analyzing the mixture by mass spectrometry; and

(c) detecting the most abundant target protein-compound conjugate that is formed, and

(d) determining the identity of the non-oligomeric organic compound present in said target protein-compound conjugate as the non-oligomeric organic compound having the greatest relative affinity for the target protein,

wherein said non-oligomeric organic compound is a novel ligand for said target protein.

59. The method of claim 58 wherein the novel ligand is less than 1500 daltons in size.

61. The method of claim 58 wherein the novel ligand is less than 750 daltons in size.

65. The method of claim 58 wherein said target protein is a TNF receptor.

81. The method of claim 58 wherein said chemically reactive group is an -SH group, a protected -SH group or an activated -SH group.

82. The method of claim 81 wherein said -SH group, protected -SH group or activated -SH group is part of a cysteine residue of said target protein.

83. The method of claim 58 wherein the library comprises at least two members.

84. The method of claim 58 wherein the library comprises at least 25 members.

85. The method of claim 58 wherein the library comprise at lest 100 members.

86. A competition assay comprising:

(a) contacting in a mixture a target protein, a reducing agent, and at least two compounds that are less than 2000 daltons and capable of forming a disulfide bond with the target protein thereby forming a target protein-compound conjugate;

(b) analyzing the mixture by mass spectrometry; and

(c) detecting the most abundant target protein-compound conjugate that is formed.

87. The assay of claim 86 further comprising determining the identify of the compound that is disulfide bonded to the target protein in the most abundant target protein-compound conjugate that is formed.

88. The assay of claim 86 wherein the compounds are less than 1500 daltons in size.

89. The assay of claim 86 wherein the compounds are less than 750 daltons in size.

93. The assay of claim 86 wherein said target protein is a TNF receptor.

95. The assay of claim 86 wherein said mixture is contacted with at least 25 compounds.

96. The assay of claim 86 wherein said mixture is contacted with at least 100 compounds.

9. EVIDENCE APPENDIX

1. WO 98/11436 (Kim *et al.*)

2. Siuzdak, G. (Mass Spectrometry for Biotechnology, New York: Academic Press, 1996, pp. 119-126).

3. WO 97/01755 (Jindal *et al.*)

4. Declaration of Gary Siuzak, Ph.D.

Items 1 and 2 were first cited by the Examiner in an Office Action mailed on December 29, 2003.

Item 3 was first cited by the Examiner in an Office Action mailed on May 25, 2005.

Item 4 was submitted by Appellants with a Preliminary Communication dated February 28, 2005.

10. RELATED PROCEEDINGS APPENDIX

None.

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(71) Applicants *(for all designated States except US)*: WHITEHEAD INSTITUTE FOR BIOMEDICAL RESEARCH [US/US]; Nine Cambridge Center, Cambridge, MA 02142 (US). THE NETHERLANDS CANCER INSTITUTE [NL/NL]; Plesmanlaan 21, NL-1066 CX Amsterdam (NL).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: KIM, Peter, S. [US/US]; 48 Baskin Road, Lexington, MA (US). SCHUMACHER, Antonius, N., M. [NL/NL]; Kenaupark 22, NL-2011 MT Haarlem (NL).

(74) Agents: GRANAHAN, Patricia et al.; Hamilton, Brook, Smith & Reynolds, P.C., Two Militia Drive, Lexington, MA 02173 (US).

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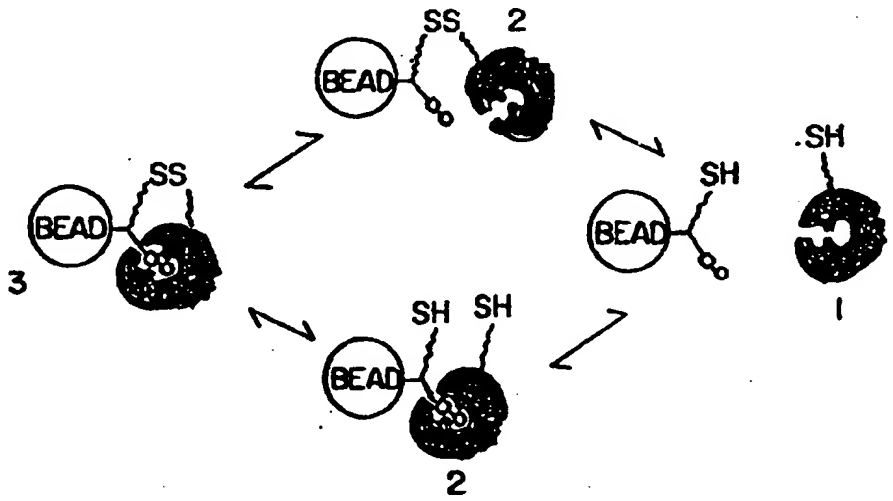
(54) Title: NON-SPECIFIC AFFINITY ENHANCEMENT TO IDENTIFY COMBINATORIAL LIBRARY MEMBERS

(57) Abstract

Non-specific affinity enhancement as a method of identifying and detecting members, such as ligands and catalysts, in a collection or library of potential ligands or catalysts, which improves the detection limits of such collections or libraries.



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NON-SPECIFIC AFFINITY ENHANCEMENT TO IDENTIFY COMBINATORIAL LIBRARY MEMBERS

Background of the Invention

Combinatorial chemistry is a tool that aids drug discovery efforts. Many different strategies for synthesizing chemicals in a combinatorial manner are available, as are many different strategies for detecting and/or identifying ligands from a combinatorial chemistry library. Unfortunately, available strategies for identifying ligands are unable to detect ligands that do not have a high affinity for the target macromolecule, which is typically a protein. Available strategies for identifying catalysts are similarly limited. As a result, combinatorial chemistry has been most useful when prior knowledge of the nature of ligands is available, so that biased combinatorial chemistry strategies can be utilized. In addition, because the strategies presently available for detecting ligands are unable to detect ligands that do not have a high affinity for a target macromolecule, combinatorial chemistry has been of limited use in identifying chemicals that bind macromolecules of pharmaceutical interest, such as chemicals that can themselves be used for therapeutic or diagnostic purposes or that can serve as lead compounds to be modified to produce a therapeutically effective agent or diagnostic reagent. It would facilitate identification of ligands and catalysts of all types, including those of pharmaceutical interest, if improved methods of detecting ligands of low affinity and catalysts were available.

Summary of the Invention

The present invention relates to a method of identifying a molecule, present in a collection or library, which binds a target molecule or which is a catalyst which acts upon a target molecule. That is, the present invention relates to a method of identifying a ligand for a target molecule and to a method of identifying a catalyst for which the target molecule is a substrate. In the present method, members of a collection or library are linked or tethered in a non-specific manner to or interact in a non-specific manner with target molecules, resulting in improved detection limits of collections or libraries (increasing the likelihood that a ligand or catalyst present in the collection or library will be identified or detected), relative to detection limits using previously-available methods. The subject invention also relates to methods of enhancing the effective concentration of a ligand for a target molecule and of enhancing the effective concentration of a catalyst which acts upon the target molecule, as well as to ligands and catalysts identified by the claimed methods. It further relates to collections or libraries of molecules (potential ligands or potential catalysts) which, as produced or as modified, contain a reactive moiety which is one member of a binding pair. Such libraries are useful in the present methods.

In one embodiment, the present invention relates to a method of identifying and/or detecting a ligand, in a collection or library of potential ligands, of a target molecule, in which potential ligands have the capacity to be linked or tethered in a non-specific manner to target molecules. The potential ligands can be linked or tethered to target molecules reversibly or irreversibly, and can be linked or tethered covalently or non-covalently. As a result, the detection limits of collections or libraries are improved, relative to detection limits using presently

available methods. The present invention also relates to methods of enhancing the effective concentration of a ligand for a target molecule and to ligands identified by the claimed methods.

One embodiment of this invention is a method of identifying or detecting a ligand for a target molecule in a library or collection of potential ligands. The method comprises creating or producing a library or collection of potential ligands which contain a reactive moiety or modifying an existing collection or library of potential ligands to contain a reactive moiety; combining the library or collection with a target molecule which contains, as obtained or as modified, a binding partner for the reactive moiety contained on the potential ligands, to produce a combination; maintaining the combination under conditions appropriate for binding of the reactive moiety and the binding partner, to produce target molecules having tethered thereto a potential ligand, and for specific binding of a target molecule with a ligand; and determining whether specific binding of a target molecule and a potential ligand tethered to the target molecule occurs. If specific binding has occurred, the potential ligand specifically bound to the target molecule is a ligand for the target molecule. Optionally, the complex of the ligand specifically bound to the target molecule can be separated or removed from the library or collection, using known methods. The ligand can also be separated from the ligand-target molecule complex, using known methods.

The binding partner and reactive moiety can be selected from a wide variety of binding pairs/partners, including free sulfhydryl groups and sulfur moieties which are available for disulfide bond formation through exchange; biotin and streptavidin/avidin; leucine zipper components; peptide-binding domains and peptides; ion chelating motifs and ions; ester bonds and other covalent

interactions; aptamers specific for caffeine and caffeine; aptamers specific for ATP and ATP; FK506 and an FK506 binding protein (FKBP); cyclosporin and cyclophilin; steroids and their respective steroid receptors; hormones and their respective hormone receptors; pharmaceutical targets and pharmaceuticals; cyclodextrins and their corresponding binding partners; antibodies and their corresponding antigens; molecules which contain, or are linked to, a magnetic force and a corresponding molecule which is attracted to it; molecules which contain, or are linked to, an electric charge, and a corresponding molecule or substance which is attracted to it; and components of charge-charge interactions. Formation of an interaction between a binding partner and a reactive moiety can be dependent on (controlled by) "cofactors", such as the formation of crosslinks between NH_2 groups that are mediated by succinimide derivatives. The interaction between a binding partner and a reactive moiety can be covalent or non-covalent.

As discussed in detail below, the potential ligands present in a collection or library, as well as the target molecule, can be any of a wide variety of molecules, including proteins (including polypeptides and peptides), oligonucleotides, DNA, RNA, protein nucleic acids, lipoproteins, glycoproteins, carbohydrates, lipids, small organic molecules, phage, viruses, toxins, drugs, membrane proteins, nucleoprotein complexes, pharmaceuticals, hormones, phosphoinositides, prostaglandins, prostacyclins, thromboxanes and large organic molecules. As is also discussed below in detail, a wide variety of linkers can be used.

The present invention permits detection or identification of ligands in collections or libraries at detection limits not possible using previously-available methods. The method, thus, can be used to identify ligands

which would not be available through use of presently-available methods. Ligands identified by the subject method have a wide variety of uses, including as drugs and reagents for therapeutic and diagnostic purposes and as lead molecules for drug design.

In one embodiment of the present invention, the effective concentration of a ligand for a target molecule is enhanced. The target molecule (a molecule for which a ligand is sought) contains, as obtained or as modified, a moiety which is one member of a binding pair. The effective concentration of a ligand for the target molecule is enhanced by creating or producing, using known methods, a collection or library of potential ligands of the target molecule, in which potential ligands contain a reactive moiety which binds the member of the binding pair contained on the target molecule. That is, the reactive moiety is one member of the binding pair whose other member is contained on the target molecule. Alternatively, an existing collection or library of potential ligands can be modified to enhance the effective concentration of a ligand for a target molecule. This is done, using known methods, by modifying library or collection components to contain a reactive moiety which binds a binding partner contained on the target molecule. Library or collection components are modified to contain one member of the binding pair; the target molecule, as obtained or as modified, contains the other (second) member of the binding pair.

A second embodiment of this invention is a method of identifying or detecting, in a library or collection of potential catalysts, a catalyst of a reaction in which the target molecule is a substrate. The method comprises creating or producing a library or collection of potential catalysts which contain a reactive moiety or modifying an existing collection or library of potential catalysts to contain a reactive moiety; combining the library or

collection with a target molecule which contains, as obtained or modified, a binding partner for the reactive moiety contained on the potential catalysts, to produce a combination; maintaining the combination under conditions appropriate for binding of the reactive moiety and the binding partner, to produce target molecules having tethered thereto a potential catalyst and for a potential catalyst to act upon the target molecule and carry out a chemical transformation; and determining whether a potential catalyst tethered to a target molecule catalyzes a reaction in which the target molecule is acted upon. If catalysis has occurred (if the target molecule is acted upon), the target molecule is a substrate of the potential catalyst (the potential catalyst is a catalyst). Alternatively, it is possible to identify a substrate for a known catalyst by screening a library of potential substrates with a target molecule which is a catalyst. The present method of identifying substrates permits detection or identification of substrates in collections or libraries at detection limits which are improved relative to detection limits using previously-known methods. In this embodiment, the linker used to tether potential catalysts to target molecules must be inert to (must not be a substrate of) the catalyst identified. Linking or tethering of a potential catalyst and a target molecule can be irreversible or reversible, and covalent or non-covalent, in this embodiment.

In this embodiment as well, the binding partner and reactive moiety can be selected from a wide variety of binding pairs/partners, including free sulfhydryl groups and sulfur moieties which are available for disulfide bond formation through exchange; biotin and streptavidin/avidin; leucine zipper components; peptide-binding domains and peptides; ion chelating motifs and ions; aptamers specific for caffeine and caffeine; aptamers specific for ATP and

ATP; FK506 and an FK506 binding protein (FKBP); cyclosporin and cyclophilin; steroids and their respective steroid receptors; hormones and their respective hormone receptors; pharmaceutical targets and pharmaceuticals; cyclodextrins and their corresponding binding partners; antibodies and their corresponding antigens; molecules which contain, or are linked to, a magnetic force and a corresponding molecule which is attracted to it; molecules which contain, or are linked to, an electric charge and a corresponding molecule or substance which is attracted to it and components of charge-charge interactions.

The potential catalysts in a collection or library, as well as the target molecule, can be any of a wide variety of molecules, including proteins (including polypeptides and peptides), oligonucleotides, DNA, RNA, protein nucleic acids, lipoproteins, glycoproteins, carbohydrates, lipids, small organic molecules, phage, viruses, toxins, drugs, membrane proteins, nucleoprotein complexes, pharmaceuticals, hormones, phosphoinositides, prostaglandins, prostacyclins, thromboxanes and large organic molecules.

The present method of identifying catalysts permits detection or identification of catalysts in collections or libraries at detection limits not possible using presently-available methods, thus making it possible to identify catalysts with lower catalytic activity than is presently possible. Such catalysts can be used, as obtained, or can be modified to produce catalysts with altered (e.g., greater or longer-lived) activity. The catalysts can be used in research and in commercial or industrial settings, such as to produce pharmaceuticals, materials such as plastics and other polymers, and other products, such as food products, detergents and other cleansers and oral hygiene products (e.g., toothpastes, mouthwashes).

Brief Description of the Drawings

Figure 1 is a schematic representation of one embodiment of the method of the present invention, in which the potential ligands are present on beads.

Figure 2 is a schematic representation of one embodiment of the method of the present invention, in which the target molecule is attached to a cell membrane.

Figure 3 is a graphic representation of results of HPLC analysis of retention time of various reaction products resulting from analysis of the effect of a second, non-specific interaction on the identification of ligands for targets of interest.

Figure 4 is a graphic representation which shows that Crk-binding peptide TS-8 preferentially forms disulfide-bonded heterodimers with the Crk SH3 under native conditions, but not under denaturing conditions.

Detailed Description of the Invention

The present invention relates to a method of improving identification or detection of a molecule, in a library or collection of molecules, which is a binding partner for a target molecule or acts catalytically upon a target molecule (which is a substrate of the molecule identified). Thus, the present invention is a method of identifying or detecting a ligand which binds a target molecule or a catalyst which acts upon a target molecule. A particular advantage of the embodiment of the present method by which ligands are identified or detected is that no prior knowledge of the ligands or of the structure of a target molecule, including the binding site, is needed. Similarly, a particular advantage of the embodiment by which catalysts are identified or detected is that it is not necessary to know anything about the catalysts or the target molecule (substrate) structure.

Identification or Detection of Ligands

In one embodiment, the present invention relates to a method of improving identification of a ligand for a target molecule, in which the ligand is present in a collection or library of potential ligands. The method is generally applicable, due to the fact that the affinity of ligands for their target molecule(s) is enhanced in a non-specific manner. As a result, ligand(s) for any selected target molecule can be identified and/or detected even though the affinity of the ligand, present in a library, for the target molecule is lower than the affinity which is necessary for identification and/or detection using presently available methods.

In the present method, a potential ligand for a target molecule is linked or tethered, in a non-specific manner, to a target molecule, thus increasing the apparent or effective concentration of the ligand for the target molecule. The non-specific binding of a potential ligand to a target molecule is effected by binding of a reactive moiety present on the potential ligand with a binding partner on the target molecule. The ligand can be linked or tethered to the target molecule covalently or non-covalently, and reversibly or irreversibly. In the embodiment in which linking is reversible, the likelihood that the ligand and target molecule will bind and remain bound under the conditions used is increased. For non-specific binding pairs for which a formed interaction is irreversible, the conditions used can be chosen such that the chance of such an interaction occurring are small in the absence of a specific interaction (e.g., by manipulation of pH, addition of untethered reactive moiety, etc.). Because a specific interaction increases the effective concentration of the two members of the binding pair, the chance of formation of an irreversible complex is increased and the formation of such complexes can be detected. An irreversible, non-specific binding pair can

also be utilized in situations where the chance of a specific interaction occurring is small in the absence of a non-specific binding interaction, provided that detection of the occurrence of a specific interaction is possible. As a result of the present invention, the detection limits of combinatorial libraries and other collections of molecules are improved (relative to detection limits using presently-available methods), for example, by 100-fold or more. Detection at millimolar affinity levels of the ligand is easily accomplished using the present invention, even in cases where there is only a single specific interaction site on the target molecule for the ligand.

Thermodynamics requires that the change or difference in ligand affinity (when tethered and untethered ligands are compared) is directly related to the free energy change for the first interaction between a potential ligand and the target molecule. For example, following the initial reaction (for example, non-specific binding of a ligand with a target molecule through the binding of members of a binding pair), each subsequent reaction (in this case, specific binding of the ligand to the target molecule to which it is tethered) is more favorable because the entropy loss is less (as compared to the entropy loss in the absence of the tether). The non-specific interaction is chosen to act in a favorable manner for binding of the ligand and its target molecule. Alternatively, if the initial reaction is specific binding of a ligand with the target molecule, the subsequent reaction (binding of the members of the binding pair) is more favorable.

For the method of the present invention to be carried out, a potential ligand and the target molecule must each contain, as obtained or as modified, a member of a binding pair, in order to permit non-specific linking or tethering of a potential ligand with the target molecule. The member of the binding pair present on a potential ligand is

referred to here as a reactive moiety or first member of a binding pair; the member present on the target molecule is referred to as a binding partner of the reactive moiety or second member of the binding pair.

Libraries of potential ligands can be produced, using known methods, in which members contain (as produced) a reactive moiety. For example, phage display libraries which comprise a single cysteine residue followed by a random sequence of amino acids can be used. As obtained, a target molecule might also include a binding partner (such as a sulfur moiety within a cysteine residue) which is available or can be made available (e.g., as a free sulfhydryl group or sulfur that is available for disulfide bond formation through exchange) for binding with a reactive moiety. If such a target molecule is used, potential ligands can be modified to include a free sulfhydryl group or a sulfur that is available for disulfide bond formation through exchange, which can be attached to the potential ligands via a linker or can be present on (attached directly to) the potential ligands. Here, non-specific binding of target molecule and potential ligands occurs through formation of a disulfide bond. Alternatively, potential ligands and target molecules can be modified to contain, respectively, a reactive moiety and a binding partner, using known methods.

In the present method of enhancing the effective concentration of a ligand for a target molecule, the target molecule contains, as it is obtained or as modified, a binding partner which is one member of a binding pair and is a binding partner for a reactive moiety present on members of a library of potential ligands for the target molecule. In the method, the target molecule containing the binding partner is combined with a library of potential ligands, in which the potential ligands contain the reactive moiety which is the second member of the binding

pair. A method of enhancing the effective concentration of a ligand for a target molecule which contains a binding molecule which is one member of a binding pair comprises modifying potential ligands in a library in such a manner that they contain the reactive moiety which is the second member of the binding pair.

In one embodiment of the present invention, the effective concentration of a ligand for a target molecule is enhanced in the following manner: the ligand is modified by the addition of a covalent, flexible linker and a reactive moiety, in such a manner that the linker is positioned between the ligand and the reactive moiety (in this embodiment, the order is ligand - linker - reactive moiety). The linker can be attached to the ligand at any point on the ligand (e.g., at either end or at an internal position). As obtained or as modified, the target molecule contains a binding partner for the reactive moiety which is attached to the ligand. As a result, the ligand and target molecule are non-specifically linked or tethered when the reactive moiety and its binding partner bind and the effective concentration of the ligand is enhanced. The effective concentration of the ligand for the target molecule is the concentration of untethered ligand that would be required, in order to obtain the same likelihood for binding between the (untethered) ligand and the target molecule, as the likelihood that the tethered ligand and target molecule will bind. Similarly, the effective concentration of the reactive moiety for the binding partner is the concentration of untethered reactive moiety required to obtain the same likelihood for binding the binding partner, as the likelihood that a tethered reactive moiety and a binding partner will bind. Because effective concentration is enhanced through use of the present method, ligands which would otherwise not be identified or detected bind the target molecule.

In one embodiment of the present method of identifying a ligand for a target molecule, a library of potential ligands is provided. In this embodiment, wherein each potential ligand has attached thereto a linker and a reactive moiety, the library can be one which, as created or produced, comprises potential ligands containing a reactive moiety or can be an existing library in which members have been modified to contain reactive moieties. The library of potential ligands is combined with a target molecule which contains a binding partner for the reactive moiety attached to the potential ligands, thereby producing a combination. The combination is maintained under conditions appropriate for binding of the reactive moiety and the binding partner and for specific binding of a target molecule with a potential ligand. The interactions which occur in the combination are represented schematically in Figure 1, in which, for illustrative purposes only, potential ligands are shown attached to beads. Initially (step 1), potential ligands and target molecules are present in the combination as separate entities. Subsequently (step 2), two interactions occur: binding of the reactive moiety on potential ligands with the binding partner on target molecules (referred to as non-specific binding) and binding of ligand(s) with the target molecule (referred to as specific binding). These interactions may occur individually (e.g., only one type of interaction occurs), sequentially (e.g., one type of interaction occurs, followed by the second type) or simultaneously. Whether the first interaction to occur between a potential ligand and a target molecule is nonspecific binding or specific binding, the result is that the effective concentration of the potential ligand (and, thus, the likelihood a ligand will bind a target molecule and remain bound) is increased. Potential ligands which bind specifically to target molecules are ligands of the

target molecule; potential ligands which do not bind target molecules are not ligands of the target molecules. The result of these interactions is a mixture, which may include any or all of the following: untethered potential ligands, untethered target molecules, tethered potential ligands, untethered ligands and tethered ligands. Specific binding of a target molecule and a potential ligand tethered thereto is determined (and, thus, a ligand of the target molecule is identified), using known methods. Once a ligand has been identified, it can be characterized. A ligand can also be separated from the complex it has formed with the target molecule. If further ligands are desired (e.g., with greater binding affinity), knowledge of the characteristics of the ligand can be used to design a biased library of potential ligands (e.g., a library of potential ligands in which a region of the ligand identified which appears to be critical for binding is varied based on characterization of the ligand identified).

The linker used in relation to the potential ligands and target molecules described herein serves the purpose of colocalization of a reactive moiety and a potential ligand and of a binding partner and a target molecule. It is only necessary that the linker be sufficiently flexible and of sufficient length to permit simultaneous specific and non-specific binding (and maintenance of bonds formed) under the conditions used. As a result, the linker can be any of a wide variety of types, including a covalent bond, a single component or unit (e.g., one amino acid), a chain of units (e.g., amino acids) joined together, a solid surface (e.g., beads or planar surfaces), and a cell membrane or cell membrane fragment. The linker can be, for example, simply a bond (e.g., a covalent bond) introduced between a component of a potential ligand and a reactive group added to the potential ligand. Alternatively, the linker can be comprised of one or more amino acids (e.g., 6-aminocaproic

acid), other organic units or a combination thereof. The amino acids can be naturally occurring (modified or unmodified) or nonnaturally occurring.

The target molecule can be attached to or present within a cell membrane (or cell membrane fragment), which serves to colocalize it with the binding partner. In this embodiment, the membrane serves as a linker. For example, a cell surface receptor for which drugs which bind are sought can be the target molecule; the receptor is present on a cell surface. (See Figure 2) If the cell used also expresses a second moiety which is a binding partner, such as FKBP, (which is attached to the cell membrane via a membrane anchor), the target molecule and binding partner (FKBP) are linked by means of the membrane. Cells or cell fragments containing both can be contacted with a collection or library of potential ligands which bear a reactive moiety which binds the binding partner on the cell membrane (here, FK-506). For example, as shown in Figure 2, the library can be comprised of potential ligands joined to FK-506 by means of a linker. Recognition and binding of FK-506 with the FKBP on the cell membrane tethers the potential ligand with the target molecule, increasing the effective concentration relative to one another and enhancing the likelihood they will bind and a ligand will be identified. Alternatively, potential ligands can be present on or attached to cell membranes or fragments, which also bear a reactive moiety, and the target molecule (bearing a binding partner) can be contacted with the cells.

The target molecule can also be contacted with potential ligands in an embodiment in which the target molecule (or molecules) is present on a chip, which also bears a binding partner, such as free sulfhydryl groups. Here, the linker between target molecule and binding partner is the chip. For example, a protein or a

collection of proteins for which a ligand(s) is sought can be used. Contacting the chip with a library of potential ligands which bear a reactive moiety results in identification of ligands, if present, in the library. Alternatively, a library of potential ligands, each of which contains a reactive moiety, can be contained on a chip, which is contacted with a target molecule containing a binding partner.

It is not necessary that both potential ligands (or catalysts) and target molecules contain a linker or if both do contain a linker, that the two linkers are of the same length or composition. It is only necessary that potential ligands and target molecules contain, respectively, a reactive moiety and a binding partner of the reactive moiety and that these two members of a binding pair are able to interact to link or tether a potential ligand to a target molecule in such a manner that the two remain joined and can interact with one another under the conditions used.

In a specific embodiment, the covalent, flexible linker is a synthetic peptide or polypeptide. The linker is a peptide which comprises a sufficient number of amino acid residues to be long enough to provide suitable space for the ligand to simultaneously interact with and bind the target molecule in a specific and non-specific manner. Attached to the peptide is a reactive group or moiety which is available to bind with a binding partner present on a target molecule. The amino acid residues in the linker can be any of the naturally-occurring amino acids (modified or unmodified), non-naturally occurring amino acids or a combination thereof. The linker can be, for example, $(glycine\text{-}glycine\text{-}serine)_n$-cysteine, where n can equal any number, provided that the linker is not so short that it prevents simultaneous specific and non-specific interaction and binding of a ligand and a target molecule. In this

embodiment, the cysteine provides a free sulfhydryl group, which is available to react with a free sulfhydryl group present on the target molecule (as obtained or as modified). The interaction of the two sulfhydryl groups results in tethering of the ligand to the target molecule and thermodynamically more favorable conditions for binding of the ligand with the target molecule. The non-specific interaction (here, of the disulfide groups) acts in a favorable manner for binding and, as a result, ligands that could not be identified in the absence of the non-specific interaction can be identified. In this embodiment, the likelihood of non-specific interaction (here, disulfide bond formation) can be varied by adjusting the concentration of external oxidizing and reducing agents (for example, oxidized and reduced glutathione, respectively) present in the solution. The direct thermodynamic relationship also provides an alternative strategy for identifying ligands from a combinatorial library; molecules that bind with higher affinity will necessarily increase the effective concentration of the other members of the binding pair to a greater extent. Thus, in this embodiment, tethered ligands that bind with higher affinity will have disulfide bonds that are more resistant to reduction by external reducing agents, such as reduced glutathione.

The library of potential ligands to be screened can be any collection or library of molecules of interest, provided that the molecules as produced contain or can be appropriately modified to contain a reactive moiety. For example, the collection or library can be a chemical library, a combinatorial chemistry library, a combinatorial biologically-encoded library (e.g., a SELEX library or a phage display library), a collection of protein variants (e.g., produced by random mutagenesis of a gene encoding the protein or produced by site-directed mutagenesis of

codons for selected residues in the protein), a cell lysate, cell culture medium, a fungal broth, a library obtained through simultaneous coupling of mixtures of building blocks, or a library formed through spatially addressable synthesis. As discussed above, the library can be present in solution or on a solid surface. If the library is on a solid surface (e.g., beads, plane), or on a cell membrane, one or more potential ligands can be present on a surface. For example, one potential ligand can be displayed per bead or a mixture or two or more potential ligands can be attached to a bead. The library of potential ligands can be an unbiased or biased library. A biased library is one which is limited or restricted to a particular set of entities or molecules and is not completely random. It includes a high frequency of molecules that contain chemical entities or building blocks which are known or thought to interact with a molecule for which a ligand is sought or contains chemical entities which resemble the structure and/or chemical properties of chemical entities known or thought to interact with a molecule for which a ligand is sought.

The nonspecific interaction between potential ligands and target molecules can occur through interactions between reactive moieties and their binding partners other than sulfhydryl groups. For example, the interactions can be those that occur between biotin and streptavidin/avidin, leucine zipper components, peptide-binding domains and peptides (e.g., SH3 domains), ion chelating motifs and ions (e.g., EF hands, His tags), DNA binding domains (e.g., zinc fingers), ester bonds and other covalent interactions, aptamers that are specific for compounds such as caffeine or ATP, and, respectively, caffeine or ATP, antibodies and their corresponding antigens, pharmaceuticals (e.g., FK506, cyclosporin), with known ligands (e.g., FKBP, cyclophilin), steroids and their respective steroid receptors, hormones

and their respective receptors, antibodies and their corresponding antigens, electric fields and charge-charge interactions. The non-specific interactions between potential ligands and target molecules can be covalent or non-covalent.

The likelihood of the non-specific interaction can be varied by use of an untethered reactive moiety or untethered binding partner. For example, in an embodiment in which the non-specific interaction occurs between biotin (tethered to the ligand) and avidin (tethered to the target molecule), the likelihood of non-specific interaction can be decreased by adding increasing concentrations of either untethered biotin and/or untethered avidin. The likelihood of non-specific interaction in this embodiment can also be decreased by dilution: at lower concentration of potential ligands and/or target molecules, the likelihood of interaction will decrease. In an embodiment in which the non-specific interaction occurs, through ester bond formation, the likelihood of non-specific interaction can be varied by altering the pH of the solution.

In one embodiment, a library of molecules (referred to as a library of potential ligands) which are to be screened for their ability to bind a target molecule is produced. Each member of the library comprises a variable region (which is the potential ligand), a linker (also referred to as a spacer) and a reactive moiety. The library of potential ligands can be attached to a solid surface (e.g., beads, plates), using known techniques, or can be maintained in solution.

In one embodiment, beads for synthesis of potential ligands are first modified to introduce a low level of sulfhydryl groups. A library that consists of potential ligands is then synthesized on these beads, using the "one-bead, one-compound" strategy. Here, the bead itself is the linker. The library is then incubated, under oxidizing

conditions, with a version of the target macromolecule containing a natural or artificial sulfhydryl group, so that a disulfide bond is formed between the target and the sulfhydryl on the bead. The beads are then washed for variable periods of time, under conditions of varying concentrations of reducing agent, followed by incubation in the presence of a sulfhydryl quenching agent, such as iodoacetate. The beads may then be washed under denaturing conditions to remove any non-covalently bound target. Beads that still contain bound target can be detected readily (for example, by antibody binding detection methods).

Target molecules for which ligands can be identified using the present method include proteins (including polypeptides and peptides), oligonucleotides, DNA, RNA, protein nucleic acids, lipoproteins, glycoproteins, carbohydrates, lipids, small organic molecules, phage, viruses, toxins, drugs, membrane proteins, nucleoprotein complexes, pharmaceuticals, hormones, phosphoinositides, prostaglandins, prostacyclins, thromboxanes and large organic molecules. A target molecule, as obtained, contains or is modified to contain a binding partner for the reactive moiety present on the potential ligands in the library to be screened. For example, in the case in which the reactive moiety present on potential ligands is a free sulfhydryl group, the target molecule will also contain or be modified to contain a free sulfhydryl group. Optionally, the target molecule can also be modified to contain a detectable moiety, useful to detect the presence of the ligand-target molecule complex formed. For example, the target molecule can be modified to include a (biotin)-(biotinylation signal). Modification of the target molecule can be an amino terminal modification, a carboxyl-terminal modification or an internal modification (modification of a non-terminal amino acid residue) if the

target molecule is a peptide, polypeptide or protein (all referred to herein as proteins). Similarly, if the target is a molecule of another type (i.e., not a protein), the modification can be a terminal (end) or internal modification.

Once specific binding of a ligand in a library of potential ligands with the target molecule has occurred, detection of the resulting complex (ligand bound to target molecule) can be carried out using known methods. Interaction of ligand and target molecule can be detected by, for example, using a target molecule which contains or is modified with an antibody epitope (e.g., SH3) containing a domain which can be identified by an antibody and/or staining or sorting with anti-tag antibodies.

The methods described herein permit the detection and identification of ligands for target molecules (such as physiologically significant mammalian proteins, biological molecules involved in diseases, small organic molecules, pharmaceuticals, toxins, proteins and oligonucleotides critical for activity of infectious organisms), particularly ligands which are not detected or identified by previously-available methods because they do not have a sufficiently high affinity for the target molecule. Ligands identified by this method are useful, for example, as drug candidates, which can be assessed, using known methods, for their ability to alter activity of the target molecule and, if necessary, can be modified to enhance their therapeutic effects. Such ligands can also be used in diagnostic methods, such as a method of diagnosing the presence of or monitoring the status of a disease with which the target molecule is associated.

Identification of Catalysts

In a further embodiment, a catalyst for (a molecule which acts catalytically upon) a target molecule is identified. In this embodiment, a random population

(collection or library) of molecules is assessed or searched for the presence of a member for which the target molecule is a catalytic substrate. The subject method makes it possible to identify molecules with weak (low) catalytic activity, which are not identifiable by previously-available methods. Here, as in the embodiment which results in identification of a ligand, members of a collection or library are tethered non-specifically to a target molecule. The linkage can be reversible or non-reversible, and covalent or non-covalent. Potential catalysts in the library or collection, as obtained or modified, bear a reactive moiety and the target molecule, as obtained or modified, bears a binding partner for the reactive moiety.

The binding partner and reactive moiety can be selected from a wide variety of binding pairs/partners, including free sulfhydryl groups and sulfur moieties which are available for disulfide bond formation through exchange; biotin and streptavidin/avidin; leucine zipper components; peptide-binding domains and peptides; ion chelating motifs and ions; ester bonds and other covalent interactions; aptamers specific for caffeine and caffeine; aptamers specific for ATP and ATP; FK506 and an FK506 binding protein (FKBP); cyclosporin and cyclophilin; steroids and their respective steroid receptors; hormones and their respective hormone receptors; pharmaceutical targets and pharmaceuticals; cyclodextrins and their corresponding binding partners; antibodies and their corresponding antigens; molecules which contain, or are linked to, a magnetic force and a corresponding molecule which is attracted to it; molecules which contain, or are linked to, an electric charge, and a corresponding molecule or substance which is attracted to it; and components of charge-charge interactions.

The potential catalysts present in a collection or library, as well as the target molecule for which a catalyst is sought can also be any of a wide variety of molecules, including proteins (including polypeptides and peptides), oligonucleotides, DNA, RNA, protein nucleic acids, lipoproteins, glycoproteins, carbohydrates, lipids, small organic molecules, phage, viruses, toxins, drugs, membrane proteins, nucleoprotein complexes, pharmaceuticals, hormones, phosphoinositides, prostaglandins, prostacyclins, thromboxanes and large organic molecules.

The library of potential catalysts used can be any collection or library of molecules, provided the molecules contain, as obtained or modified, a reactive moiety which binds a binding partner present on the target molecule used. For example, the collection or library can be a chemical library, a combinatorial chemistry library, a combinatorial biologically-encoded library (e.g., a SELEX library or a phage display library), a collection of protein variants (e.g., produced by random mutagenesis of a gene encoding the protein or produced by site-directed mutagenesis of codons for selected residues in the protein), a cell lysate, cell culture medium, a fungal broth, a library obtained through simultaneous coupling of mixtures of building blocks, or a library formed through spatially addressable synthesis. As discussed above, the library can be present in solution or on a solid surface. If the library is on a solid surface (e.g., beads, plane), or on a cell membrane, one or more potential catalysts can be present on a surface. For example, one potential catalyst can be displayed per bead or a mixture of two or more potential catalysts can be attached to a bead.

When the library of potential catalysts which contain the reactive moiety is combined with the target molecule which contains the binding partner and the resulting

combination is maintained under appropriate conditions (for binding of the reactive moiety and the binding partner and for a catalytic reaction to occur in which the target molecule is acted upon and a chemical transformation occurs), potential catalysts become tethered to the target molecule, thus enhancing the effective concentration of a catalyst in the library and the target molecule (substrate). Catalytic activity, thus, is more likely to occur than is the case with presently available methods.

Catalytic activity (which indicates that a catalyst has been identified) can be assessed or shown by a variety of methods. For example, if a catalyst for a reaction which results in cleavage of a target molecule which is a protein (e.g., angiotensin) is desired, the target protein can be labelled by addition of a fluorescent group (fluorophore) at one location (e.g., at one end) and addition of a fluorescence quencher at another location (e.g., at the other end). For example, a bead or other solid surface can be used as the linker and the linking carried out such that the fluorophore remains attached to the target protein after cleavage of the protein occurs (e.g., into two fragments). Cleavage of the protein in a reaction catalyzed by a member of the library results in removal of the peptide fragment which bears the fluorescence quencher. Therefore, although the beads bearing the labelled target protein were initially non-fluorescent (due to the presence of the quencher), they become fluorescent if a catalyst is present in the library or collection (as a result of the catalyst's acting upon the target protein).

In another embodiment, the library assessed can be a library of protein variants, such as a library of variants of a protein, in which the variants retain the ability to fold in a manner similar to that of the wild type (nonvariant or "parent") protein. For example, the library

can be a collection of variants of GB1 (a small 56 - residue protein which includes loops), which contain a combinatorial assortment of amino acid residues in the loops of the protein. The substrate in this embodiment is a peptide to be cleaved. As above, the peptide is labelled with a fluorophore and a fluorescence quencher, typically one at each end of the peptide. The linker, which is a bead or other solid surface, is attached to the peptide such that after cleavage occurs, the fluorophore remains attached to the peptide fragment that remains attached to the bead or solid surface, but the quencher has been removed. That cleavage has occurred (and, thus, the presence of a catalyst) is easily demonstrated by fluorescence in the combination produced when the library and target molecule are combined and maintained under conditions appropriate for linking of potential catalysts with the target peptide and cleavage of the peptide. Fluorescence is shown by shining a light of appropriate wavelength on the combination. The GB1 variants on fluorescent beads can be sequenced to determine which members of the library are catalytically active.

Other detection methods can also be used, such as colorimetric and antibody - based methods.

The present invention is illustrated by the following examples, which are not intended to be limiting in any way.

EXAMPLE 1: Isolation of Ligands for the Crk N-SH3 Domain Through Non-specific Affinity Enhancement

The following is a description of identification of ligands in a collection or library in which ligands are covalently attached to a solid surface (beads) during synthesis.

The NH_2-terminal SH3 domain of the c-Crk protein is a 58 amino acid domain that lacks cysteine residues. The 3-

dimensional structure of a complex of this domain with a peptide ligand has been solved by X-ray crystallography. To identify novel ligands for the NH_2-terminal SH3 domain of the c-Crk protein, using non-specific affinity enhancement the following experiment may be performed:

A). Derivatives of the NH_2-terminal Crk SH3 domain with a COOH-terminal linker and free sulfhydryl, and an NH_2-terminal biotin/biotinylation signal are generated by conventional molecular biological and protein expression methods.

(1): (biotin)-(biotinylation signal)-(c-Crk residue 134-191)-$(Gly\text{-}Gly\text{-}Ser)_3$-Cys

(2): (biotin)-(biotinylation signal)-(c-Crk residue 134-191)-$(Gly\text{-}Gly\text{-}Ser)_4$-Cys

Synthetic linkers with the sequence $(Gly\text{-}Gly\text{-}Ser)_3$-Cys and $(Gly\text{-}Gly\text{-}Ser)_4$-Cys are produced by solid phase peptide synthesis.

B). Beads for solid phase peptide synthesis are modified by coupling of a substochiometric amount of sidechain-protected N-acetylated cysteine (e.g., 1:5 ratio of cysteine to reactive groups). A library of synthetic compounds is subsequently produced on these beads using cycles of split and pool synthesis, using FMOC-protected monomer building blocks as described (K.S. Lam et al., Nature, 1991; A.P. Combs et al., JACS, 1996).

C). The biotinylated SH3 domain derivatives and the corresponding synthetic linkers (SH3:linker;1:10) are incubated with the library of compounds, in Tris buffer (10 mM, pH 7.5), in the presence of a redox system (e.g., reduced glutathione (GSH) and oxidized glutathione (GSSG) at various ratios). The inclusion of an excess of the corresponding (unbiotinylated) synthetic linkers is optional and is used to select against compounds that interact with the linker, rather than with the SH3 domain. The beads are subsequently washed with buffer under

increasingly reducing conditions. The reaction is subsequently quenched by the addition of acetic acid (final concentration of 5%), and the beads are washed with a denaturing buffer (6 M Guanidine-HCl/5% HAc) to remove any non-covalently bound SH3 domain. The beads are then washed with 10 mM Tris pH 7.5/100 mM iodoacetamide and incubated with streptavidin-alkaline phosphatase. The presence of bound SH3 domain is subsequently determined by visual inspection of the beads under a light microscope. Beads that are positive are collected and the identity of the compounds on these beads is determined by microsequencing.

EXAMPLE 2: Demonstration of the Effect of a Non-Specific Interaction on the Identification of Ligands for Targets of Interest

To demonstrate the effect if a second non-specific interaction on the identification of ligands for targets of interest, the following experiment was performed. A derivative of the NH2-terminal c-Crk SH3 domain with a NH2-terminal Ha-tag and a C COOH-terminal (GGS)3 linker, followed by a free cysteine residue was incubated with a derivative of a Crk SH3 ligand with an NH2-terminal linker and free cysteine residue. Disulfide bond formation between the two free cysteine residues in the presence or absence of denaturant was monitored by reverse phase HPLC. The sequences of the two derivatives are, respectively: amino-AYPYDVPDYASAEYVRALFDFNGNDEEDLPFKKGDILRIRDK-PEEQWWNAEDSEGKRGMIPVPYVEKYRGGSGGSGGSC-carboxyl and acetyl-CGGSGGSPPPALPPKKR-carboxyl.

Figure 3 indicates the retention times of the various reaction products on c-18 reverse phase HPLC. Figure 4 indicates that the Crk-binding peptide TS-8 preferentially forms disulfide-bonded heterodimers with the Crk SH3 domain under native conditions, but not under denaturing

conditions. This experiment illustrates that under conditions where a specific interaction between a target molecule and ligand can take place, preferential formation of disulfide-medicated ligand-target heterodimers can be observed.

Reaction conditions: Peptide and SH3 domain (200 and 50 μM respectively) were incubated under anaerobic conditions for 18-24 hours in 100 mM Tris (pH 8.7)/200 mM KCl/1 mM EDTA/1 mM GSSG (glutathione dimer) in the presence of absence of 8M urea. The reaction was terminated by the addition of 5% HAc (final concentration) and the reaction products were quantitated by reverse phase HPLC on a Waters C-18 column.

EXAMPLE 3: Isolation of Ligands Through Non-covalent Affinity Enhancement

The following is a description of identification of ligands in a collection or library, in which ligands are covalently attached to a solid surface (beads) during synthesis. In this example the second interaction is formed through non-covalent interaction: heterodimerization of the pACID and pBASE leucine zippers.

A). A derivative of the NH3 terminal SH3 domain of c-Crk is generated through conventional molecular biology/protein expression techniques.

(1): (Myc tag) - (c-Crk 134-191) - ((GGS)4) - (pACID) (pACID = AQLEKELQALEKENAQLEWELQALEKELAQ, see O'Shea *et al.*, (1993) *Curr. Biol.* 3:658); (Myc tag = EQKLISEEDL) see Boehringer Mannheim product information, pi1667149-12195 and Evan, G.I. *et al.*, *Mol. Cell. Biol.* 5:3610-3616 (1985)/

B). A library of synthetic compounds is generated for instance using cycles of split and pool synthesis (Lam, Nature 1991), with the following structure.

(2): (randomized stretch) - ((GGS(2) - (pBASE)

(pBASE = AQLKKKLQALKKKNAQLKWKLQALKKKLAQ, see O'Shea *et al.*, *Curr. Biol.* 3:658 (1993))

C). The library of compounds is incubated with the Crk derivative to reveal compounds that interact with this SH3 domain. In this assay the presence of bound SH3 domain is visualized using alkaline phosphatase labeled anti-myc tag antibody (Boehringer Mannheim) by visual inspection of the beads under a light microscope. Anti-c-myc recognizes the 9E10 epitope (sequence EQKLISEEDL), which was derived from the human c-myc protein. Beads that are positive are collected and microsequenced. The inclusion of an excess of free pBASE or pACID peptide is optional and can be used to increase the stringency of the assay.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

CLAIMS

We claim:

1. A method of enhancing the effective concentration of a ligand for a target molecule, wherein the target molecule, as obtained or as modified, contains one member of a binding pair, comprising creating a collection or library of potential ligands for the target molecule, wherein the library is comprised of potential ligands which contain a reactive moiety which is the second member of the binding pair.

2. The method of Claim 1 wherein the binding partner and the reactive moiety are each a free sulfhydryl group or a sulfur moiety which is available for disulfide bond formation through exchange.

3. The method of Claim 1 wherein the binding partner and the reactive moiety are members of a binding pair selected from the group consisting of:
   a) biotin and streptavidin/avidin;
   b) leucine zipper components;
   c) peptide-binding domains and peptides;
   d) ion chelating motifs and ions;
   e) covalent interactions;
   f) aptamers specific for caffeine and caffeine;
   g) aptamers specific for ATP and ATP;
   h) FK506 and an FK506 binding protein (FKBP);
   I) cyclosporin and cyclophilin;
   j) steroid receptors and steroids;
   k) hormone receptors and hormones;
   l) pharmaceutical targets and pharmaceuticals;
   m) cyclodextrins and their corresponding binding partners;

n) antibodies and their corresponding antigens;
o) molecules which contain, or are linked to, a magnetic force and a corresponding molecule which is attracted to it;
p) molecules which contain, or are linked to, an electric charge and a molecule that is attracted to it; and
q) charge-charge interactions.

4. A method of enhancing the effective concentration of a ligand for a target molecule which, as obtained or as modified, contains a binding partner which is one member of a binding pair and is a binding partner for a reactive moiety in a library of potential ligands for the target molecule, comprising modifying potential ligands in the library in such a manner that they contain the reactive moiety which is the second member of the binding pair.

5. The method of Claim 4 wherein the binding partner and the reactive moiety are each a free sulfhydryl group or a sulfur moiety which is available for disulfide bond formation through exchange.

6. The method of Claim 4 wherein the binding partner and the reactive moiety are members of binding pairs selected from the group consisting of:
a) biotin and streptavidin/avidin;
b) leucine zipper components;
c) peptide-binding domains and peptides;
d) ion chelating motifs and ions;
e) covalent interactions;
f) aptamers specific for caffeine and caffeine;
g) aptamers specific for ATP and ATP;
h) FK506 and an FK506 binding protein (FKBP);

i) cyclosporin and cyclophilin;
j) steroid receptors and steroids;
k) hormone receptors and hormones;
l) pharmaceutical targets and pharmaceuticals;
m) cyclodextrins and their corresponding binding partners;
n) antibodies and their corresponding antigens;
o) molecules which contain, or are linked to, a magnetic force and a corresponding molecule which is attracted to it;
p) molecules which contain, or are linked to, an electric charge and a molecule that is attracted to it; and
q) charge-charge interactions.

7. A method of enhancing the effective concentration of a ligand for a target molecule, wherein the target molecule as obtained or as modified contains a binding partner, comprising modifying the ligand by the addition of a linker and a reactive moiety which binds the binding partner contained on the target molecule, such that the linker is positioned between the ligand and the reactive moiety.

8. The method of Claim 7 wherein the linker is selected from the group consisting of:
a) covalent bonds
b) single units;
c) chains of units;
d) solid surfaces;
e) cell membranes; and
f) cell membrane fragments.

9. The method of Claim 7 wherein the binding partner and the reactive moiety are each a free sulfhydryl group or a sulfur moiety which is available for disulfide bond formation through exchange.

10. The method of Claim 7 wherein the binding partner and the reactive moiety are members of binding pairs selected from the group consisting of:

a) biotin and streptavidin/avidin;
b) leucine zipper components;
c) peptide-binding domains and peptides;
d) ion chelating motifs and ions;
e) covalent interactions;
f) aptamers specific for caffeine and caffeine;
g) aptamers specific for ATP and ATP;
h) FK506 and an FK506 binding protein (FKBP);
i) cyclosporin and cyclophilin;
j) steroid receptors and steroids;
k) hormone receptors and hormones;
l) pharmaceutical targets and pharmaceuticals;
m) cyclodextrins and their corresponding binding partners;
n) antibodies and their corresponding antigens;
o) molecules which contain, or are linked to, a magnetic force and a corresponding molecule which is attracted to it;
p) molecules which contain, or are linked to, an electric charge and a molecule that is attracted to it; and
q) charge-charge interactions.

11. The method of Claim 1 wherein the target molecule is selected from the group consisting of: proteins, oligonucleotides, DNA, RNA, protein nucleic acids, lipoproteins, glycoproteins, carbohydrates, lipids,

small organic molecules, phage, viruses, toxins, drugs, membrane proteins, nucleoprotein complexes, pharmaceuticals, hormones, phosphoinositides, prostaglandins, prostacyclins, thromboxanes and large organic molecules.

12. The method of Claim 4 wherein the target molecule is selected from the group consisting of: proteins, oligonucleotides, DNA, RNA, protein nucleic acids, lipoproteins, glycoproteins, carbohydrates, lipids, small organic molecules, phage, viruses, toxins, drugs, membrane proteins, nucleoprotein complexes, pharmaceuticals, hormones, phosphoinositides, prostaglandins, prostacyclins, thromboxanes and large organic molecules.

13. The method of Claim 7 wherein the target molecule is selected from the group consisting of: proteins, oligonucleotides, DNA, RNA, protein nucleic acids, lipoproteins, glycoproteins, carbohydrates, lipids, small organic molecules, phage, viruses, toxins, drugs, membrane proteins, nucleoprotein complexes, pharmaceuticals, hormones, phosphoinositides, prostaglandins, prostacyclins, thromboxanes and large organic molecules.

14. A method of identifying a ligand for a target molecule, in a library of potential ligands, comprising the steps of:

a) producing a library of potential ligands wherein the potential ligands contain a reactive moiety;

b) combining the library of potential ligands with a target molecule which contains, as obtained or modified, a binding partner for the reactive

moiety contained on the potential ligands, thereby producing a combination;

c) maintaining the combination under conditions appropriate for binding of the reactive moiety and the binding partner to produce target molecules having tethered thereto a potential ligand and for specific binding of a target molecule with a ligand; and

d) determining whether specific binding of a target molecule and a potential ligand tethered thereto occurs, wherein if specific binding has occurred, the potential ligand specifically bound to the target molecule is a ligand for the target molecule.

15. The method of Claim 14 wherein the binding partner and the reactive moiety are each a free sulfhydryl group or a sulfur moiety which is available for disulfide bond formation through exchange.

16. The method of Claim 14 wherein the binding partner and the reactive moiety are members of binding pairs selected from the group consisting of:

a) biotin and streptavidin/avidin;
b) leucine zipper components;
c) peptide-binding domains and peptides;
d) ion chelating motifs and ions;
e) covalent interactions;
f) aptamers specific for caffeine and caffeine;
g) aptamers specific for ATP and ATP; and
h) FK506 and an FK-506 binding partner;
i) cyclosporin and cyclosporin;
j) steroid receptors and steroids;
k) hormone receptors and hormones;
l) pharmaceutical targets and pharmaceuticals;

m) cyclodextrins and their corresponding binding partners;

n) antibodies and their corresponding antigens;

o) molecules which contain, or are linked to, a magnetic force and a corresponding molecule which is attracted to it;

p) molecules which contain, or are linked to, an electric charge and a molecule that is attracted to it; and

q) charge-charge interactions.

17. The method of Claim 14 wherein the target molecule is selected from the group consisting of: proteins, oligonucleotides, DNA, RNA, protein nucleic acids, lipoproteins, glycoproteins, carbohydrates, lipids, small organic molecules, phage, viruses, toxins, drugs, membrane proteins, nucleoprotein complexes, pharmaceuticals, hormones, phosphoinositides, prostaglandins, prostacyclins, thromboxanes and large organic molecules.

18. A method of identifying, in a library of potential ligands, a ligand for a target molecule, comprising the steps of:

a) creating a library of potential ligands, wherein each potential ligand has attached thereto a linker and a reactive moiety, wherein the linker is positioned between the ligand and the reactive moiety;

b) combining the library of potential ligands with a target molecule which contains a binding partner for the reactive moiety attached to the potential ligands, thereby producing a combination;

c) maintaining the combination under conditions appropriate for binding of the reactive moiety

and the binding partner to produce target molecules having tethered thereto a potential ligand and for specific binding of a target molecule with a potential ligand; and

d) determining whether specific binding of a target molecule and a potential ligand occurs, wherein if specific binding has occurred, the potential ligand specifically bound to the target molecule is a ligand for the target molecule.

19. The method of Claim 18 wherein the linker is selected from the group consisting of:

a) covalent bonds
b) single units;
c) chains of units;
d) solid surfaces;
e) cell membranes; and
f) cell membrane fragments.

20. The method of Claim 18 wherein the binding partner and the reactive moiety is each a free sulfhydryl group or a sulfur moiety which is available for disulfide bond formation through exchange.

21. The method of Claim 18 wherein the binding partner and the reactive moiety are members of binding pairs selected from the group consisting of:

a) biotin and streptavidin/avidin;
b) leucine zipper components;
c) peptide-binding domains and peptides;
d) ion chelating motifs and ions;
e) covalent interactions;
f) aptamers specific for caffeine and caffeine;
g) aptamers specific for ATP and ATP; and
h) FK506 and an FK-506 binding partner;

i) cyclosporin and cyclophilin;
j) steroid receptors and steroids;
k) hormone receptors and hormones;
l) pharmaceutical targets and pharmaceuticals;
m) cyclodextrins and their corresponding binding partners;
n) antibodies and their corresponding antigens;
o) molecules which contain, or are linked to, a magnetic force and a corresponding molecule which is attracted to it;
p) molecules which contain, or are linked to, an electric charge and a molecule that is attracted to it; and
q) charge-charge interactions.

22. The method of Claim 18 wherein the target molecule is selected from the group consisting of: proteins, oligonucleotides, DNA, RNA, protein nucleic acids, lipoproteins, carbohydrates, glycoproteins, small organic molecules, pharmaceuticals, hormones, phosphoinositides, prostaglandins, prostacyclins, thrombokinase, membrane proteins, nucleoprotein complexes, phage, viruses, large organic molecules, and toxins.

23. The method of Claim 18 wherein the potential ligands are present on a solid surface.

24. The method of Claim 18 wherein the solid surface is beads.

25. The method of Claim 24 wherein more than one potential ligand is present on each bead.

26. The method of Claim 24 wherein a single potential ligand is present on each bead.

27. The method of Claim 18 wherein the target molecule additionally has attached thereto a detectable moiety.

28. The method of Claim 27 wherein the detectable moiety is a biotin-biotinylation signal or an antibody epitope.

29. The method of Claim 18 wherein the potential ligands are present on a cell membrane.

30. A method of enhancing the effective concentration of a ligand for a target molecule, wherein the target molecule, as obtained or modified, contains one member of a binding pair, comprising modifying the ligand to contain a reactive moiety which is the second member of the binding pair.

31. A method of enhancing the effective concentration of a catalyst for a target molecule, wherein the target molecule, as obtained or as modified, contains one member of a binding pair, comprising creating a collection or library of potential catalysts for the target molecule, wherein the library is comprised of potential catalysts which contain a reactive moiety which is the second member of the binding pair.

32. The method of Claim 31 wherein the binding partner and the reactive moiety are each a free sulfhydryl group or a sulfur moiety which is available for disulfide bond formation through exchange.

33. The method of Claim 31 wherein the binding partner and the reactive moiety are members of a binding pair selected from the group consisting of:

a) biotin and streptavidin/avidin;
b) leucine zipper components;
c) peptide-binding domains and peptides;
d) ion chelating motifs and ions;
e) covalent interactions;
f) aptamers specific for caffeine and caffeine;
g) aptamers specific for ATP and ATP;
h) FK506 and an FK506 binding protein (FKBP);
i) cyclosporin and cyclophilin;
j) steroid receptors and steroids;
k) hormone receptors and hormones;
l) pharmaceutical targets and pharmaceuticals;
m) cyclodextrins and their corresponding binding partners;
n) antibodies and their corresponding antigens;
o) molecules which contain, or are linked to, a magnetic force and a corresponding molecule which is attracted to it;
p) molecules which contain, or are linked to, an electric charge and a molecule that is attracted to it; and
q) charge-charge interactions.

34. A method of enhancing the effective concentration of a catalyst for a target molecule which, as obtained or as modified, contains a binding partner which is one member of a binding pair and is a binding partner for a reactive moiety in a library of potential catalysts for the target molecule, comprising modifying potential catalysts in the library in such a manner that they contain the reactive moiety which is the second member of the binding pair.

35. The method of Claim 34 wherein the binding partner and the reactive moiety are each a free sulfhydryl group or a sulfur moiety which is available for disulfide bond formation through exchange.

36. The method of Claim 34 wherein the binding partner and the reactive moiety are members of binding pairs selected from the group consisting of:

a) biotin and streptavidin/avidin;
b) leucine zipper components;
c) peptide-binding domains and peptides;
d) ion chelating motifs and ions;
e) covalent interactions;
f) aptamers specific for caffeine and caffeine;
g) aptamers specific for ATP and ATP;
h) FK506 and an FK506 binding protein (FKBP);
i) cyclosporin and cyclophilin;
j) steroid receptors and steroids;
k) hormone receptors and hormones;
l) pharmaceutical targets and pharmaceuticals;
m) cyclodextrins and their corresponding binding partners;
n) antibodies and their corresponding antigens;
o) molecules which contain, or are linked to, a magnetic force and a corresponding molecule which is attracted to it;
p) molecules which contain, or are linked to, an electric charge and a molecule that is attracted to it; and
q) charge-charge interactions.

37. A method of enhancing the effective concentration of a catalyst for a target molecule, wherein the target molecule as obtained or as modified contains a binding partner, comprising modifying the catalyst by the

addition of a linker and a reactive moiety which binds the binding partner contained on the target molecule, such that the linker is positioned between the catalyst and the reactive moiety.

38. The method of Claim 37 wherein the linker is selected from the group consisting of:
a) covalent bonds
b) single units;
c) chains of units;
d) solid surfaces;
e) cell membranes; and
f) cell membrane fragments.

39. The method of Claim 37 wherein the binding partner and the reactive moiety are each a free sulfhydryl group or a sulfur moiety which is available for disulfide bond formation through exchange.

40. The method of Claim 37 wherein the binding partner and the reactive moiety are members of binding pairs selected from the group consisting of:
a) biotin and streptavidin/avidin;
b) leucine zipper components;
c) peptide-binding domains and peptides;
d) ion chelating motifs and ions;
e) covalent interactions;
f) aptamers specific for caffeine and caffeine;
g) aptamers specific for ATP and ATP;
h) FK506 and an FK506 binding protein (FKBP);
i) cyclosporin and cyclophilin;
j) steroid receptors and steroids;
k) hormone receptors and hormones;
l) pharmaceutical targets and pharmaceuticals;

m) cyclodextrins and their corresponding binding partners;

n) antibodies and their corresponding antigens;

o) molecules which contain, or are linked to, a magnetic force and a corresponding molecule which is attracted to it;

p) molecules which contain, or are linked to, an electric charge and a molecule that is attracted to it; and

q) charge-charge interactions.

41. The method of Claim 31 wherein the target molecule is selected from the group consisting of: proteins, oligonucleotides, DNA, RNA, protein nucleic acids, lipoproteins, glycoproteins, carbohydrates, lipids, small organic molecules, phage, viruses, toxins, drugs, membrane proteins, nucleoprotein complexes, pharmaceuticals, hormones, phosphoinositides, prostaglandins, prostacyclins, thromboxanes and large organic molecules.

42. The method of Claim 34 wherein the target molecule is selected from the group consisting of: proteins, oligonucleotides, DNA, RNA, protein nucleic acids, lipoproteins, glycoproteins, carbohydrates, lipids, small organic molecules, phage, viruses, toxins, drugs, membrane proteins, nucleoprotein complexes, pharmaceuticals, hormones, phosphoinositides, prostaglandins, prostacyclins, thromboxanes and large organic molecules.

43. The method of Claim 37 wherein the target molecule is selected from the group consisting of: proteins, oligonucleotides, DNA, RNA, protein nucleic acids, lipoproteins, glycoproteins, carbohydrates, lipids,

small organic molecules, phage, viruses, toxins, drugs, membrane proteins, nucleoprotein complexes, pharmaceuticals, hormones, phosphoinositides, prostaglandins, prostacyclins, thromboxanes and large organic molecules.

44. A method of identifying a catalyst for a target molecule, in a library of potential catalysts, comprising the steps of:

a) producing a library of potential catalysts wherein the potential catalysts contain a reactive moiety;

b) combining the library of potential catalysts with a target molecule which contains, as obtained or modified, a binding partner for the reactive moiety contained on the potential catalysts, thereby producing a combination;

c) maintaining the combination under conditions appropriate for binding of the reactive moiety and the binding partner to produce target molecules having tethered thereto a potential catalyst and for a potential catalyst to act upon the target molecule and carry out a chemical transformation; and

d) determining whether a catalytic reaction occurs in which a catalyst acts upon the target molecule and carries out a chemical transformation, wherein if such a catalytic reaction occurs, the potential catalyst is a catalyst for the target molecule.

45. The method of Claim 44 wherein the binding partner and the reactive moiety are each a free sulfhydryl group or a sulfur moiety which is available for disulfide bond formation through exchange.

46. The method of Claim 44 wherein the binding partner and the reactive moiety are members of binding pairs selected from the group consisting of:

a) biotin and streptavidin/avidin;
b) leucine zipper components;
c) peptide-binding domains and peptides;
d) ion chelating motifs and ions;
e) covalent interactions;
f) aptamers specific for caffeine and caffeine;
g) aptamers specific for ATP and ATP;
h) FK506 and an FK-506 binding partner;
i) cyclosporin and cyclosporin;
j) steroid receptors and steroids;
k) hormone receptors and hormones;
l) pharmaceutical targets and pharmaceuticals;
m) cyclodextrins and their corresponding binding partners;
n) antibodies and their corresponding antigens;
o) molecules which contain, or are linked to, a magnetic force and a corresponding molecule which is attracted to it;
p) molecules which contain, or are linked to, an electric charge and a molecule that is attracted to it; and
q) charge-charge interactions.

47. The method of Claim 44 wherein the target molecule is selected from the group consisting of: proteins, oligonucleotides, DNA, RNA, protein nucleic acids, lipoproteins, glycoproteins, carbohydrates, lipids, small organic molecules, phage, viruses, toxins, drugs, membrane proteins, nucleoprotein complexes, pharmaceuticals, hormones, phosphoinositides, prostaglandins, prostacyclins, thromboxanes and large organic molecules.

48. A method of identifying, in a library of potential catalysts, a catalyst for a target molecule, comprising the steps of:

a) creating a library of potential catalysts, wherein each potential catalyst has attached thereto a linker and a reactive moiety, wherein the linker is positioned between the catalyst and the reactive moiety;

b) combining the library of potential catalysts with a target molecule which contains a binding partner for the reactive moiety attached to the potential catalysts, thereby producing a combination;

c) maintaining the combination under conditions appropriate for binding of the reactive moiety and the binding partner to produce target molecules having tethered thereto a potential catalyst and for a potential catalyst to act upon the target molecule and carry out a chemical transformation; and

d) determining whether a catalytic reaction occurs in which a catalyst acts upon the target molecule and carries out a chemical transformation, wherein if such a catalytic reaction occurs, the potential catalyst is a catalyst for the target molecule.

49. The method of Claim 48 wherein the linker is selected from the group consisting of:

a) covalent bonds

b) single units;

c) chains of units;

d) solid surfaces;

e) cell membranes; and

f) cell membrane fragments.

50. The method of Claim 48 wherein the binding partner and the reactive moiety is each a free sulfhydryl group or a sulfur moiety which is available for disulfide bond formation through exchange.

51. The method of Claim 48 wherein the binding partner and the reactive moiety are members of binding pairs selected from the group consisting of:

a) biotin and streptavidin/avidin;
b) leucine zipper components;
c) peptide-binding domains and peptides;
d) ion chelating motifs and ions;
e) covalent interactions;
f) aptamers specific for caffeine and caffeine;
g) aptamers specific for ATP and ATP;
h) FK506 and an FK-506 binding partner;
i) cyclosporin and cyclophilin;
j) steroid receptors and steroids;
k) hormone receptors and hormones;
l) pharmaceutical targets and pharmaceuticals;
m) cyclodextrins and their corresponding binding partners;
n) antibodies and their corresponding antigens;
o) molecules which contain, or are linked to, a magnetic force and a corresponding molecule which is attracted to it;
p) molecules which contain, or are linked to, an electric charge and a molecule that is attracted to it; and
q) charge-charge interactions.

52. The method of Claim 48 wherein the target molecule is selected from the group consisting of: proteins, oligonucleotides, DNA, RNA, protein nucleic acids, lipoproteins, glycoproteins, carbohydrates, lipids,

small organic molecules, phage, viruses, toxins, drugs, membrane proteins, nucleoprotein complexes, pharmaceuticals, hormones, phosphoinositides, prostaglandins, prostacyclins, thromboxanes and large organic molecules.

53. The method of Claim 48 wherein the potential catalysts are present on a solid surface.

54. The method of Claim 48 wherein the solid surface is beads.

55. The method of Claim 54 wherein more than one potential catalyst is present on each bead.

56. The method of Claim 54 wherein a single potential catalyst is present on each bead.

57. The method of Claim 48 wherein the target molecule additionally has attached thereto a detectable moiety.

58. The method of Claim 47 wherein the detectable moiety is a fluorophore.

59. The method of Claim 58 wherein the potential catalysts are present on a cell membrane.

60. A collection or library of potential ligands produced by the method of Claim 1.

61. A collection or library of potential ligands produced by the method of Claim 31.

62. The method of Claim 1 wherein the potential ligand is selected from the group consisting of: proteins, oligonucleotides, DNA, RNA, protein nucleic acids, lipoproteins, glycoproteins, carbohydrates, lipids, small organic molecules, phage, viruses, toxins, drugs, membrane proteins, nucleoprotein complexes, pharmaceuticals, hormones, phosphoinositides, prostaglandins, prostacyclins, thromboxanes and large organic molecules.

63. The method of Claim 4 wherein the potential ligand is selected from the group consisting of: proteins, oligonucleotides, DNA, RNA, protein nucleic acids, lipoproteins, glycoproteins, carbohydrates, lipids, small organic molecules, phage, viruses, toxins, drugs, membrane proteins, nucleoprotein complexes, pharmaceuticals, hormones, phosphoinositides, prostaglandins, prostacyclins, thromboxanes and large organic molecules.

64. The method of Claim 7 wherein the potential ligand is selected from the group consisting of: proteins, oligonucleotides, DNA, RNA, protein nucleic acids, lipoproteins, glycoproteins, carbohydrates, lipids, small organic molecules, phage, viruses, toxins, drugs, membrane proteins, nucleoprotein complexes, pharmaceuticals, hormones, phosphoinositides, prostaglandins, prostacyclins, thromboxanes and large organic molecules.

65. The method of Claim 34 wherein the potential catalyst is selected from the group consisting of: proteins, oligonucleotides, DNA, RNA, protein nucleic acids, lipoproteins, glycoproteins, carbohydrates, lipids, small organic molecules, phage, viruses, toxins,

drugs, membrane proteins, nucleoprotein complexes, pharmaceuticals, hormones, phosphoinositides, prostaglandins, prostacyclins, thromboxanes and large organic molecules.

66. The method of Claim 35 wherein the potential catalyst is selected from the group consisting of: proteins, oligonucleotides, DNA, RNA, protein nucleic acids, lipoproteins, glycoproteins, carbohydrates, lipids, small organic molecules, phage, viruses, toxins, drugs, membrane proteins, nucleoprotein complexes, pharmaceuticals, hormones, phosphoinositides, prostaglandins, prostacyclins, thromboxanes and large organic molecules.

67. The method of Claim 37 wherein the potential catalyst is selected from the group consisting of: proteins, oligonucleotides, DNA, RNA, protein nucleic acids, lipoproteins, glycoproteins, carbohydrates, lipids, small organic molecules, phage, viruses, toxins, drugs, membrane proteins, nucleoprotein complexes, pharmaceuticals, hormones, phosphoinositides, prostaglandins, prostacyclins, thromboxanes and large organic molecules.

68. A library comprising potential ligands for a target molecule, wherein potential ligands each contain a reactive moiety which is one member of a binding pair, the binding pair selected from the group consisting of:

a) biotin and streptavidin/avidin;
b) leucine zipper components;
c) peptide-binding domains and peptides;
d) ion chelating motifs and ions;
e) covalent interactions;

f) aptamers specific for caffeine and caffeine;
g) aptamers specific for ATP and ATP;
h) FK506 and an FK506 binding protein (FKBP);
i) cyclosporin and cyclophilin;
j) steroid receptors and steroids;
k) hormone receptors and hormones;
l) pharmaceutical targets and pharmaceuticals;
m) cyclodextrins and their corresponding binding partners;
n) antibodies and their corresponding antigens;
o) molecules which contain, or are linked to, a magnetic force and a corresponding molecule which is attracted to it;
p) molecules which contain, or are linked to, an electric charge and a molecule that is attracted to it; and
q) charge-charge interactions.

69. A library of Claim 68 wherein the ligands are selected from the group consisting of: proteins (including polypeptides and peptides), oligonucleotides, DNA, RNA, protein nucleic acids, lipoproteins, glycoproteins, carbohydrates, lipids, small organic molecules, phage, viruses, toxins, drugs, membrane proteins, nucleoprotein complexes, pharmaceuticals, hormones, phosphoinositides, prostaglandins, prostacyclins, thromboxanes and large organic molecules.

70. A library comprising potential catalysts of a target molecule, wherein potential catalysts each contain a reactive group which is one member of a binding pair, the binding pair selected from the group consisting of:
a) biotin and streptavidin/avidin;

b) leucine zipper components;
c) peptide-binding domains and peptides;
d) ion chelating motifs and ions;
e) covalent interactions;
f) aptamers specific for caffeine and caffeine;
g) aptamers specific for ATP and ATP;
h) FK506 and an FK506 binding protein (FKBP);
i) cyclosporin and cyclophilin;
j) steroid receptors and steroids;
k) hormone receptors and hormones;
l) pharmaceutical targets and pharmaceuticals;
m) cyclodextrins and their corresponding binding partners;
n) antibodies and their corresponding antigens;
o) molecules which contain, or are linked to, a magnetic force and a corresponding molecule which is attracted to it;
p) molecules which contain, or are linked to, an electric charge and a molecule that is attracted to it; and
q) charge-charge interactions.

71. A library of Claim 70 wherein the catalysts are selected from the group consisting of: proteins, oligonucleotides, DNA, RNA, protein nucleic acids, lipoproteins, glycoproteins, carbohydrates, lipids, small organic molecules, phage, viruses, toxins, drugs, membrane proteins, nucleoprotein complexes, pharmaceuticals, hormones, phosphoinositides, prostaglandins, prostacyclins, thromboxanes and large organic molecules.

72. The method of Claim 23 wherein more than one potential ligand is present on each bead.

73. The method of Claim 29 wherein the potential ligands are present on a cell membrane.

74. The method of Claim 29 wherein one potential ligand is present on a cell membrane.

75. The method of Claim 29 wherein more than one potential ligand is present on a cell membrane.

76. The method of Claim 53 wherein more than one potential catalyst is present on each bead.

77. The method of Claim 53 wherein a single potential catalyst is present on each bead.

78. The method of Claim 59 wherein more than one potential catalyst is present on each bead.

79. The method of Claim 59 wherein a single potential catalyst is present on each bead.

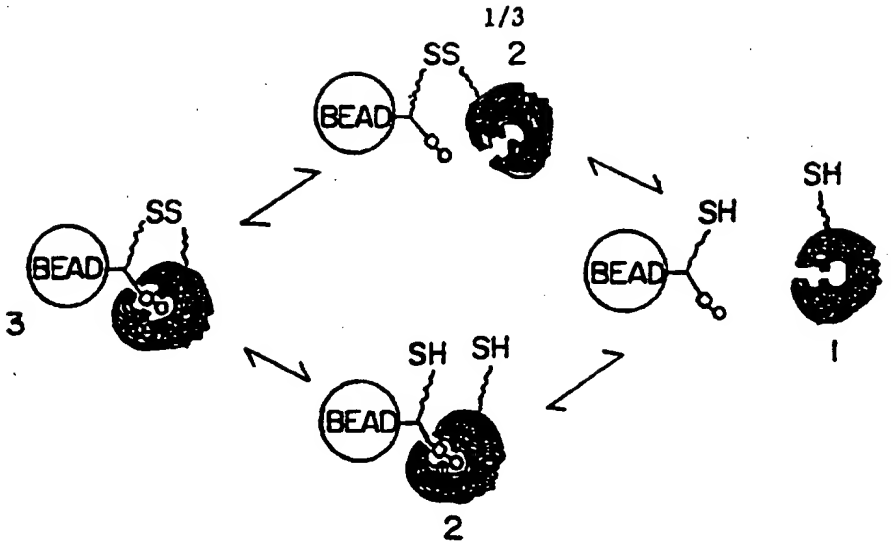
80. The method of Claim 14 wherein the potential ligand is selected from the group consisting of: proteins, oligonucleotides, DNA, RNA, protein nucleic acids, lipoproteins, glycoproteins, carbohydrates, lipids, small organic molecules, phage, viruses, toxins, drugs, membrane proteins, nucleoprotein complexes, pharmaceuticals, hormones, phosphoinositides, prostaglandins, prostacyclins, thromboxanes and large organic molecules.

81. The method of Claim 18 wherein the potential ligand is selected from the group consisting of: proteins, oligonucleotides, DNA, RNA, protein nucleic acids, lipoproteins, glycoproteins, carbohydrates, lipids,

small organic molecules, phage, viruses, toxins, drugs, membrane proteins, nucleoprotein complexes, pharmaceuticals, hormones, phosphoinositides, prostaglandins, prostacyclins, thromboxanes and large organic molecules.

82. The method of Claim 44 wherein the potential catalyst is selected from the group consisting of: proteins, oligonucleotides, DNA, RNA, protein nucleic acids, lipoproteins, glycoproteins, carbohydrates, lipids, small organic molecules, phage, viruses, toxins, drugs, membrane proteins, nucleoprotein complexes, pharmaceuticals, hormones, phosphoinositides, prostaglandins, prostacyclins, thromboxanes and large organic molecules.

83. The method of Claim 48 wherein the potential catalyst is selected from the group consisting of: proteins, oligonucleotides, DNA, RNA, protein nucleic acids, lipoproteins, glycoproteins, carbohydrates, lipids, small organic molecules, phage, viruses, toxins, drugs, membrane proteins, nucleoprotein complexes, pharmaceuticals, hormones, phosphoinositides, prostaglandins, prostacyclins, thromboxanes and large organic molecules.


1/3
SS
2
BEAD
SH
SH
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SS
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3
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SH
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1
2

FIG. 1

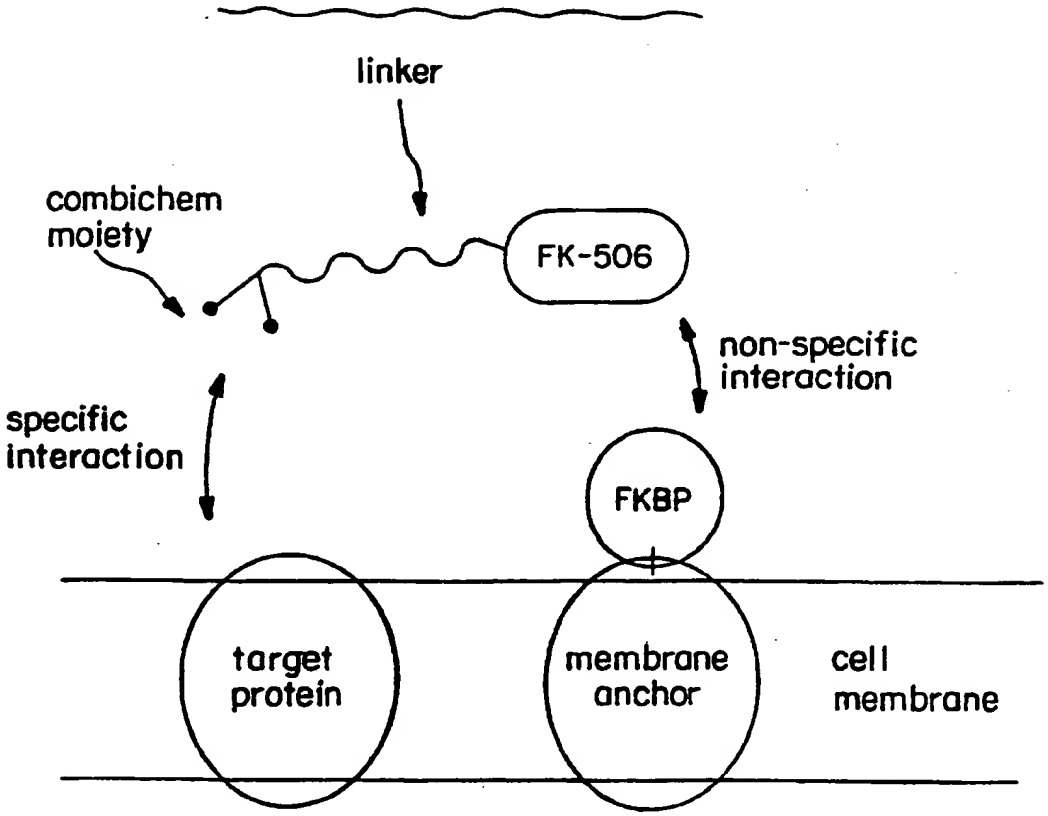
linker
combichem moiety
FK-506
non-specific interaction
specific interaction
FKBP
target protein
membrane anchor
cell membrane

FIG. 2

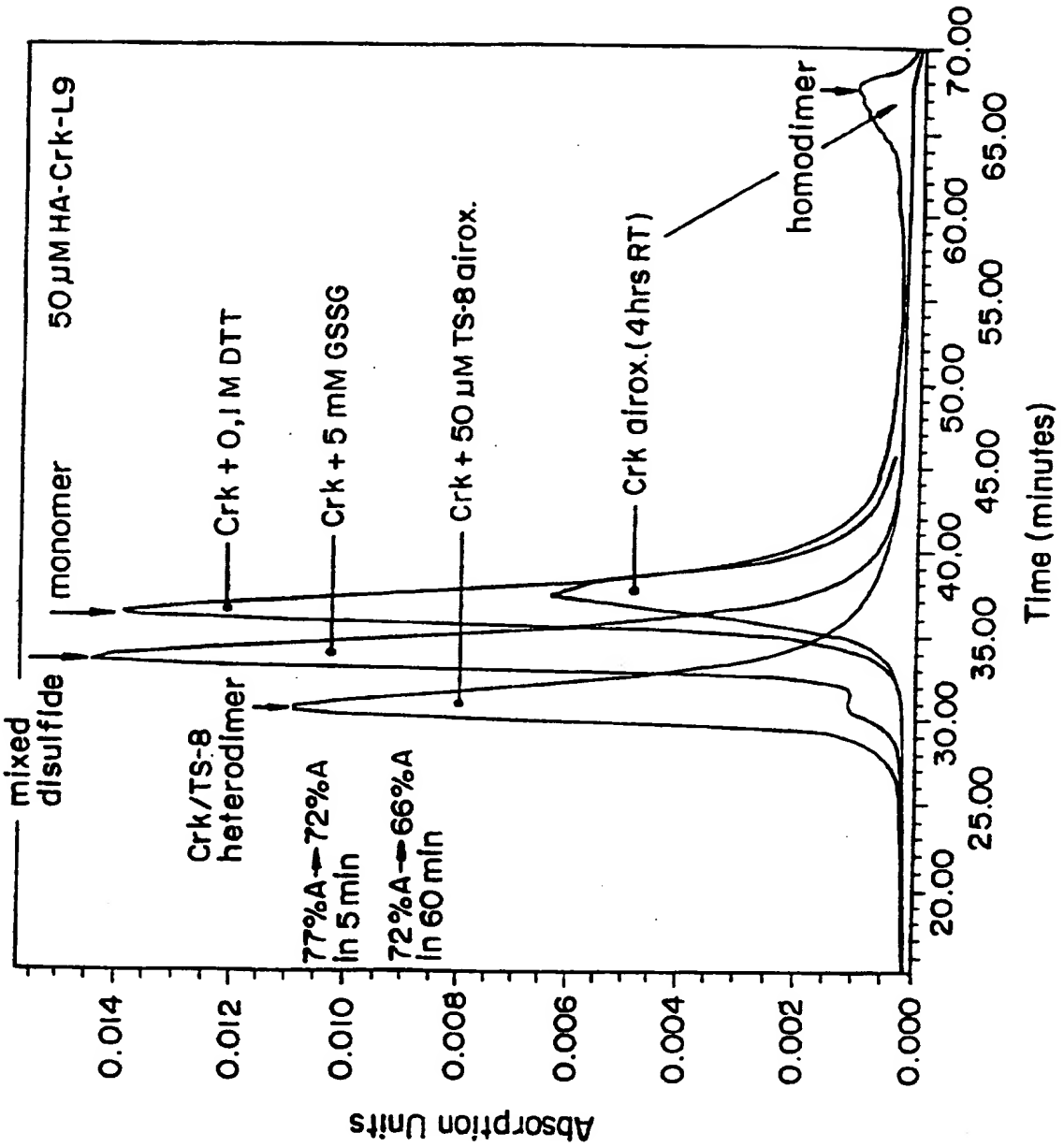
50 µM HA-Crk-L9
monomer
Crk + 0,1 M DTT
Crk + 5 mM GSSG
Crk + 50 µM TS-8 airox.
Crk airox.(4hrs RT)
homodimer
mixed disulfide
Crk/TS-8 heterodimer
77%A→72%A in 5 min
72%A→66%A in 60 min
Absorption Units
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
20.00
25.00
30.00
35.00
40.00
45.00
50.00
55.00
60.00
65.00
70.00
Time (minutes)

FIG. 3

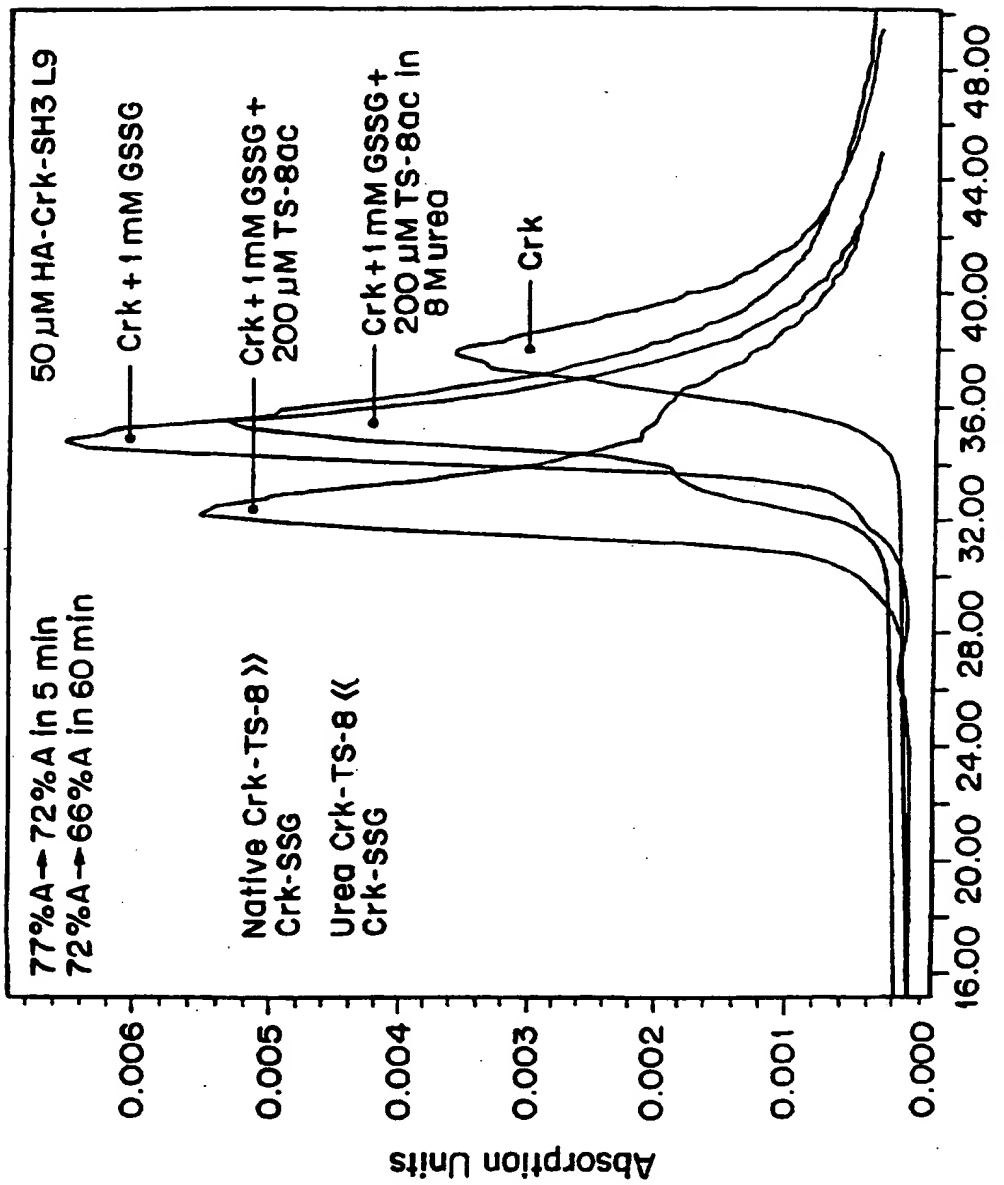
50 µM HA-Crk-SH3 L9
77%A→72%A in 5 min
72%A→66%A in 60 min
Crk + 1 mM GSSG
Crk + 1mM GSSG + 200 µM TS-8ac
Crk+1 mM GSSG + 200 µM TS-8ac in 8 M urea
Crk
Native Crk-TS-8 »
Crk-SSG
Urea Crk-TS-8 «
Crk-SSG
Absorption Units
0.000
0.001
0.002
0.003
0.004
0.005
0.006
16.00 20.00 24.00 28.00 32.00 36.00 40.00 44.00 48.00
Time (minutes)

FIG. 4

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/16424

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/53 G01N33/543 G01N33/546

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	EP 0 801 307 A (CHUGAI BIOPHARMACEUTICALS INC) 15 October 1997 Whole document	1-83
E	WO 97 43302 A (HOLDINGBOLAGET VID GOETEBORGS ;AHLBERG PER (SE); BALTZER LARS (SE)) 20 November 1997 see claims 1-6 see page 5, line 7 - page 9, line 25	1-83
E	WO 97 35202 A (UNIV PRINCETON) 25 September 1997 see claims 1-19 see page 20, line 25 - page 21, line 19	1-83

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24 February 1998	04/03/1998

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,A	WO 97 15831 A (SANGSTAT MEDICAL CORP ;LUSSOW ALEXANDER R (US); BUELOW ROLAND (US)) 1 May 1997 whole document	1-83
P,A	WO 97 00267 A (PENCE INC) 3 January 1997 Whole document	1-83
P,A	WO 96 41004 A (UNIV CALIFORNIA) 19 December 1996 Whole document	1-83

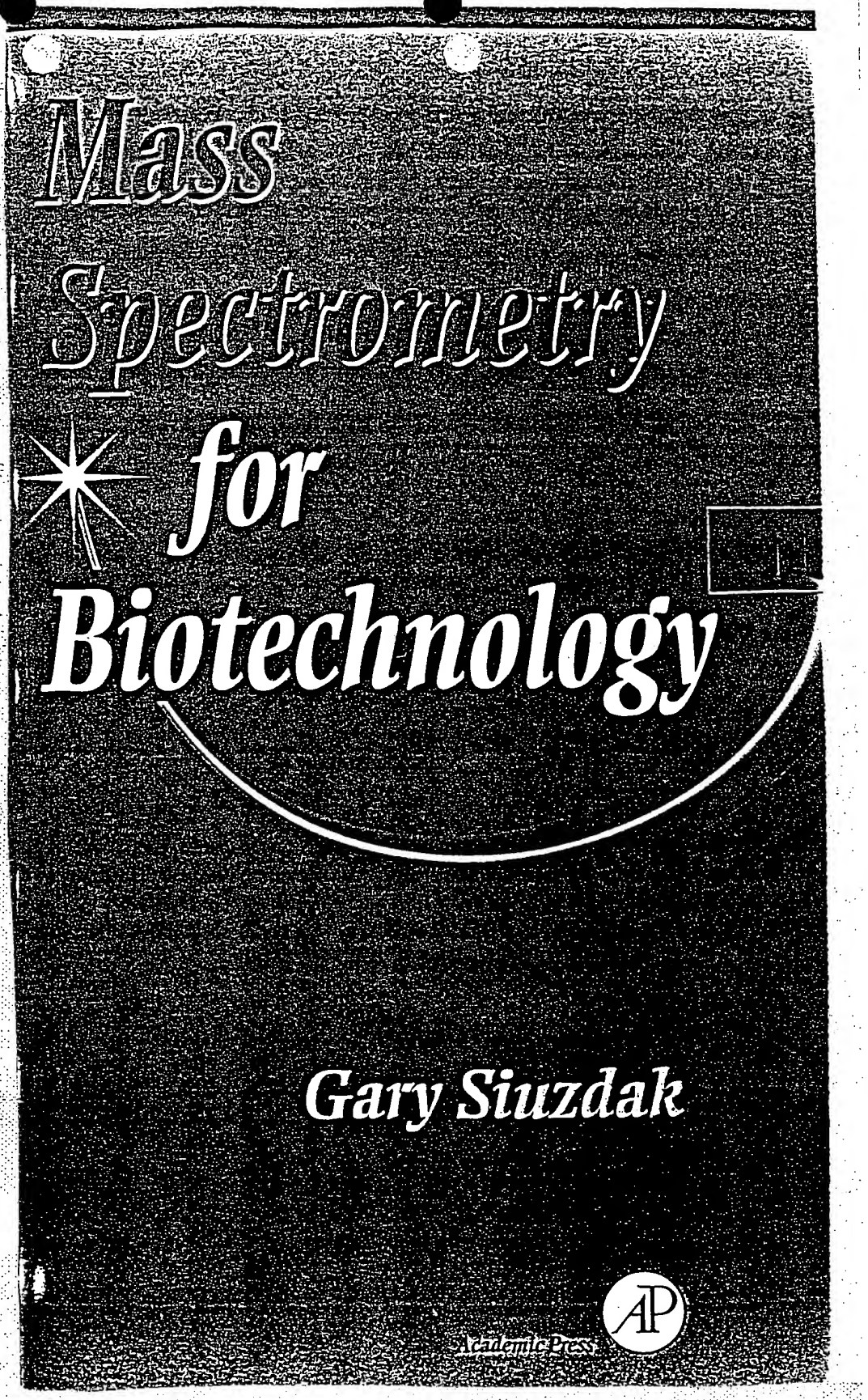
1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter. .onal Application No
PCT/US 97/16424

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0801307 A	15-10-97	AU 2661997 A	22-10-97
		WO 9737220 A	09-10-97
WO 9743302 A	20-11-97	NONE	
WO 9735202 A	25-09-97	AU 2341397 A	10-10-97
WO 9715831 A	01-05-97	AU 7168996 A	15-05-97
		CA 2207760 A	01-05-97
		EP 0799422 A	08-10-97
WO 9700267 A	03-01-97	AU 6118196 A	15-01-97
WO 9641004 A	19-12-96	NONE	



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CHAPTER 6

Specific Applications

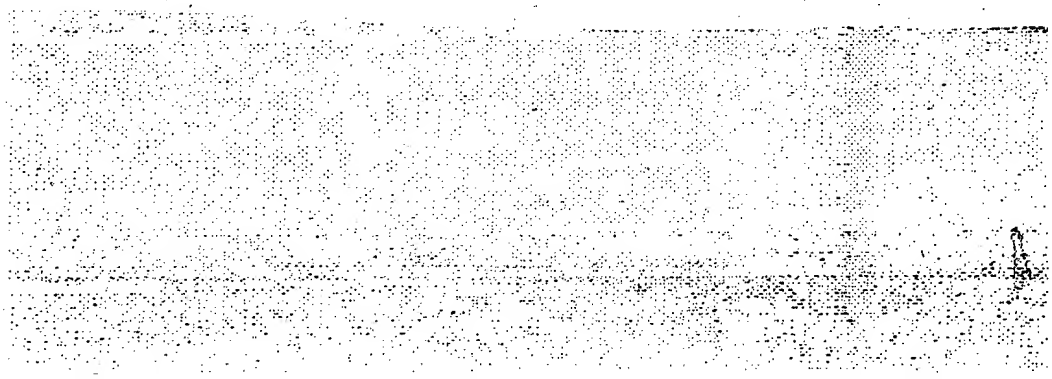
Don't let your bias keep you from doing a good experiment—Richard A. Lerner and Stephen B. H. Kent

Throughout the first five chapters the capabilities which mass spectrometry has to offer were discussed. I would like to change course from surveying the capabilities to how it is being applied to real biochemical issues. In the foregoing a cursory look at what is possible with the new generation of mass spectrometers, I have not delved too deeply into any one subject. The following final chapter is less nebulous. Presented here are condensed versions of six studies, each section being the summary of one or more papers describing unique applications of mass spectrometry.

Chemical Characterization of a Family of Brain Lipids with Sleep–Inducing Potential

The concept that there are endogeneous compounds associated with sleep has a long and complicated history, during which a large variety of substances have been proposed. Applying the sensitivity of modern analytical techniques to the study of sleep, a fresh, purely chemical approach was taken in attempting to identify molecules of the central nervous system.

Specifically, the sleep–wake states of feline subjects were examined in the following study. Cerebral spinal fluid (CSF) was removed from the feline subjects and then, using liquid chromatography, its components were separated. Electrospray tandem mass spectrometry, gas chromatography–mass spectrometry (GC–MS), and thin-layer chromatography (TLC) were also employed in the analysis of the CSF, along with infrared spectroscopy (IR), nuclear magnetic resonance spectroscopy (NMR), and chemical degradation procedures. The goal was to identify new molecules associated with the sleep–wake cycle.



CSF analysis began with preparative liquid chromatography fraction collection. These experiments produced UV data on each fraction to determine any differences between the felines at various points in their sleep cycle. An absorbance was found to be particularly prominent in the CSF of cats that were kept awake for an extended period of time (18 hr).

Even though the compound associated with this absorbance was only present in small amounts, partial characterization was initially obtained by performing exact mass measurements and tandem mass analysis. Using an API III Perkin Elmer SCIEX triple-quadrupole mass spectrometer, electrospray mass analysis on the fractions associated with the differences in the chromatogram produced a significant ion at *m/z* 282. That was determined to be the MH^+ ion, and an exact mass determination on the unknown compound by FAB (Fisons/VG ZAB-VSE) was consistent with the molecular formula $C_{18}H_{35}NO$.

CID was used to perform MS^2 and MS^3 experiments on the ion at *m/z* 282. Tandem mass analysis (Figure 6.1) at *m/z* 282 revealed a distinct fragmentation pattern in the low-molecular-mass range, consistent with other long-chain alkanes. Neutral losses of 17 and 35 Da from the parent ion indicated a loss of ammonia followed by water. Performing additional MS^3 experiments on the daughter ions at *m/z* 265 and 247 revealed that the daughter ion at 265 fragmented to form the granddaughter ion at 247. This suggested that the ion at 247 was the result of sequential losses (loss of 17 Da $\{NH_3\}$ followed by 18 Da $\{H_2O\}$), as opposed to a neutral loss independent of the daughter ion at 265. Additional deuterium exchange experiments were consistent with at least two protons on this molecule being exchangeable.

On the basis of these experiments, compounds that best corresponded to the data were synthesized and the resullts obtained on these compounds

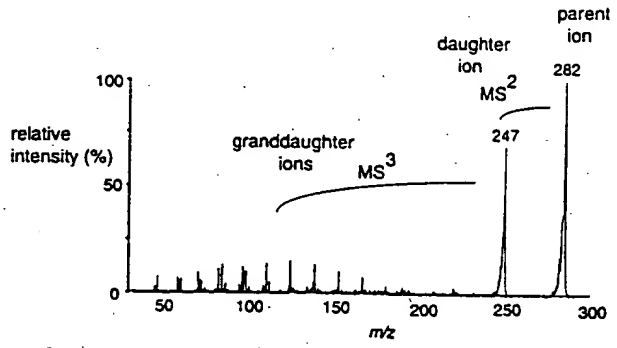


Figure 6.1 Mass spectral data (MS, MS^2, and MS_3) obtained on sleep-inducing substance.

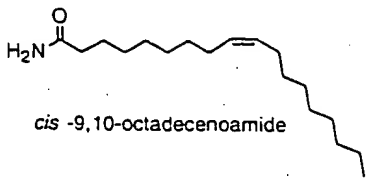


Figure 6.2 Sleep-inducing substance isolated from spinal fluid.

eventually led to the speculation that the unknown was *cis*-9,10-octadecenoamide (Figure 6.2). Additional natural compound was obtained for NMR, IR, and chemical degradation studies.

Chemical degradation techniques were first employed on synthetic fatty acid amides, identifying ozonolysis as conducive to the analysis of these agents. GC–MS analysis of the ozonolysis reaction mixture derived from the natural lipid revealed nonyl aldehyde as the only C-terminal aldehyde present. Nonyl aldehyde corresponds to an olefin positioned seven methylenes away from the terminal methyl group of the alkyl chain, which in the case of a C18 fatty acid primary amide, is the 9,10 position. The NMR and IR spectra of *cis*-9,10-octadecenoamide and the natural compound were also found to be identical.

Thus, at the conclusion of this effort, with the employment of mass spectrometry, GC, GC–MS, TLC, IR, NMR, and ozonolysis, the exact structure of the endogenous lipid, including the position and configuration of its olefin, was unambiguously determined to be *cis*-9,10-octadecenoamide.

Following its identification, synthetic *cis*-9,10-octadecenoamide was injected into rats in order to test its effect. It induced a marked, long-lasting motor quiescence and an eyes-closed, sedated behavior characteristic of normal sleep. Further studies on *cis*-9,10-octadecenoamide and other compounds observed in the CSF are currently being performed. It is interesting how, by allowing these analyses to be done, analytical technology is broadening the traditional view of lipid molecules as passive structural elements of cellular architecture and is increasing awareness of the active roles these agents play in transducing cell signals and modifying cell behavior.

Final confirmation of the unknown's identity was obtained by independent synthesis and comparison of the spectral data. The results are summarized in Table 6.1.

References

Cravatt, B. F., Prospero-Garcia, O., Siuzdak, G., Gilula, N. B., Henriksen, S. J., Boger, D. L., and Lerner, R. A. (1995). *Chemical characterization of a family of brain lipids with sleep inducing potential. Science* **268**, 1506–1509.

TABLE 6.1
Analysis of Sleep-Inducing Brain Lipid from Cats

Data type	Summary of data
Exact mass	$[C_{18}H_{35}NO + Na]^+$ Observed 304.2614 Actual 304.2616
Deuterium exchange	*m/z* 285 $[M-2H^+ + 3D^+]^+$, at least two exchangeable protons.
Isotope	Isotope pattern at *m/z* = 282 indicates that it is not a multiply charged species, nor does it have any elements with an unusual isotopic pattern (e.g. Cl or Br).
Cationization	Electrospray MS observation of *m/z* 304 $[M + Na]^+$ and *m/z* 320 $[M + K]^+$ confirms that M = 281.
MS^2 data	Fragment ion *m/z* 265 corresponding to $[MH - NH_3]^+$. Fragment ion *m/z* 247 corresponding to $[MH - NH_3 - H_2O]^+$. Lipid fragment ions.
MS^3 data	Fragmentation of *m/z* 265 ⩾ *m/z* 247 (sequential loss) suggests the *m/z* 247 fragment is related to the *m/z* 265 fragment ion. Lipid fragment ions.
NMR data	Confirms lipid portion of molecule and configuration of double bond.
Ozonolysis with GC–MS	Identified the location of the double bond.
IR data	Confirms identity of unknown as *cis* isomer.

Note. Data from Cravatt *et al.* (1995) and Lerner *et al.* (1994).

Lerner, R. A., Siuzdak, G., Prospero-Garcia, O., Henriksen, S. J., Boger, D. L., and Cravatt, B. F. (1994). *Cerebrodiene: A new brain lipid isolated from sleep deprived cats. Proc. Natl. Acad. Sci. USA* **91**, 9505–9508.

Monitoring Antibody Catalysis

The following section highlights two examples using mass spectrometry to monitor antibody and enzyme catalysis. In the studies described, natural enzymes and designed catalytic antibodies were used in conjunction with electrospray mass spectrometry to study the analysis of noncovalent binding between an antibody and its antigen. Antibody–substrate reaction intermediates were also observed.

Antibodies are components of the basic defense mechanism in our immune system, their formation being an immunological response to the presence of foreign substances (antigens). In general, the interaction of an antibody with an antigen results in the noncovalent formation of an antigen/antibody complex. An antibody elicited by a specific antigen (hapten) exhibits a shape and charge complementary to that hapten. This was exploited by chemists to design so-called catalytic antibodies capable of catalyzing specific chemical transformations. Catalytic antibodies are produced by introducing into mice haptens that structurally resemble a reaction's transition state species. Antibodies produced by accordingly immunized mice may, in effect, catalyze the reaction by binding and stabilizing the true metastable transition state which the hapten was meant to mimic.

In this first study, a single-chain catalytic antibody was used with a molecular weight of 26,419.9 Da. Previously, the antibody was found to bind tightly to the hapten shown in Figure 6.3. (Antibodies generally exhibit high affinities (<1 nM) for the haptens they were elicited with.) Our first effort was toward observing the antibody/hapten complex with electrospray mass spectrometry.

As described in Chapter 4, the electrospray ionization source allows for an ion's kinetic energy to be adjusted through the declustering potential. Declustering potentials on the order of 70 V or greater usually promote the dissociation of noncovalent complexes as well as covalent fragmentation, while lower potentials (<70 V) are conducive to the observation of noncovalent complexes (protein complexes have been analyzed at declustering potentials of 40 V). In this case, the antibody/hapten complex was observed even at declustering potentials greater than 130 V. Complete dissociation was observed at a declustering potential of 175 V where the

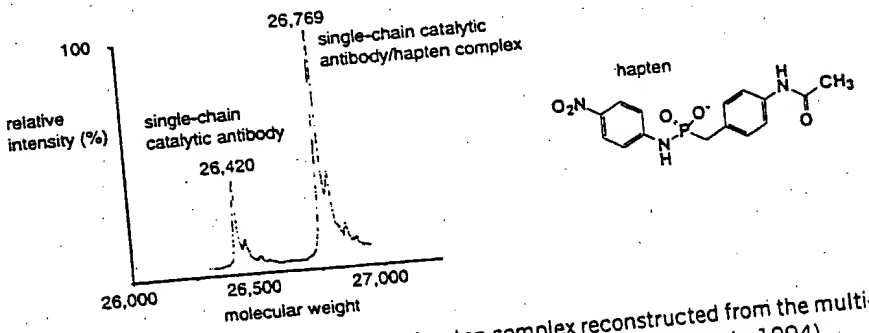


Figure 6.3 The noncovalent antibody–hapten complex reconstructed from the multiply charged states of the mass spectra. (Adapted from Siuzdak *et al.*, 1994).

antibody peak was still present, but the complex was no longer observed, consistent with its noncovalent nature. The considerably weaker binding of an inhibitor *p*-nitroaniline (k_d = 10 μM) was indicated by the complete lack of any observable complex formation in the mass spectrum even at declustering potentials of 40 V (data not shown).

Another interesting observation in these experiments was the apparent change in the charge state distribution that occurred upon addition of the hapten. The mass spectrum of the antibody prior to hapten addition showed the presence of the charge states between 18+ and 12+. However, only two charge states, 12+ and 13+, were observed for the hapten/antibody complex at the same declustering potential (Figure 6.4). The altered charge state distribution is believed to reflect global conformational changes and proton displacement by the hapten at the binding site.

The same catalytic antibody was used to study the hydrolysis of an anilide for which this antibody was designed (Figure 6.5). Electrospray is particularly well-suited for examining covalent reaction intermediates of nucleophilic catalysis, since it can be performed at acidic pH, where hydroxide-mediated reactions are minimized. Although this technique cannot be used for the intact catalytic antibody, because of its high molecular mass (~150 kDa), it is ideally suited for examining smaller fragments such as the single-chain Fv fragment.

In this experiment the antibody was first analyzed alone, and then in the presence of substrate (Figure 6.5). Upon addition of substrate, a peak consistent with an adduct mass of 26,666 Da was observed. The observed mass increase of 247 Da is identical (within error) to the mass of the acyl functionality of the substrate. These spectra were acquired with declustering potentials of 175–250 V, under conditions where even high-affinity ($\leq$ nM) noncovalent complexes are separated. Additional experiments with an antibody mutant, devoid of catalytic activity, showed no accumulation of

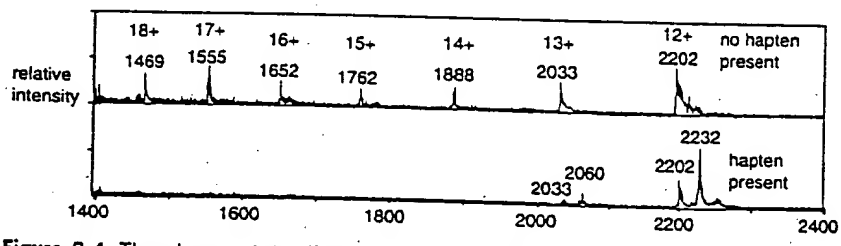


Figure 6.4 The charge state distribution of the SCA was observed under identical conditions before and after addition of the hapten. (Adapted from Siuzdak *et al.*, 1994).

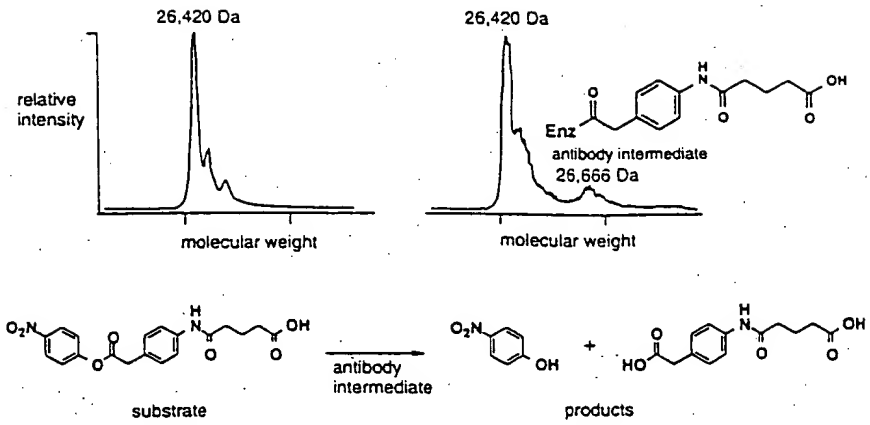


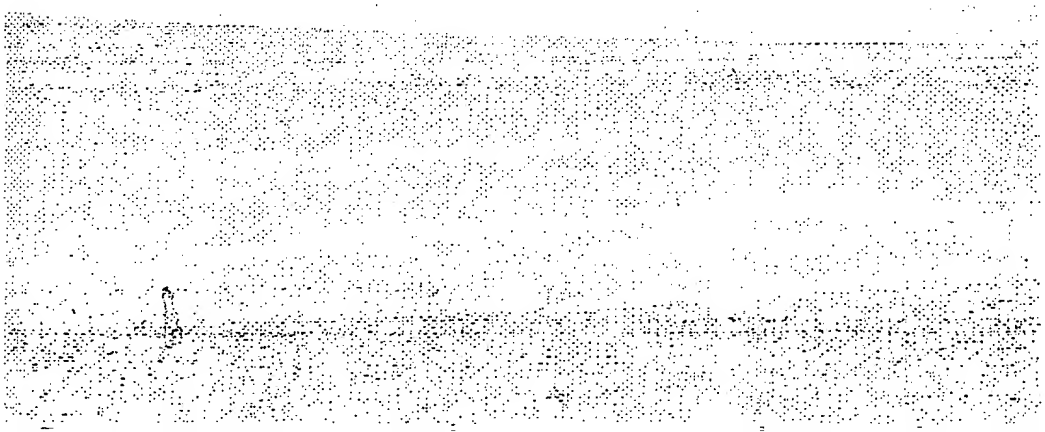
Figure 6.5 Single-chain catalytic antibody without (left) and with the substrate (right). In the presence of substrate, the formation of a covalent intermediate with the antibody can be observed. (Adapted from Krebs *et al.*, 1995).

the intermediate. These experiments provided the first direct evidence for the acyl–antibody intermediate previously proposed for this antibody.

The reaction described previously, enzyme-catalyzed ester hydrolysis, is a fundamental biochemical process. The use of fluorescence or absorption spectroscopic techniques often facilitates the kinetic study of these enzyme-catalyzed reactions. However, fluorescence or absorption spectroscopy requires that the substrate of the product have a fluorescing or absorbing chromophore. In cases in which this requirement is not fulfilled, the study of enzymes and catalytic antibodies can be severely limited. Electrospray mass spectrometry has also been used for the screening and characterization of antibodies with lipase activity since it has proven capable of analyzing proteins and small molecules with high accuracy and offers the possibility of observing compounds on the basis of their ability to be ionized, independent of the presence of chromophore.

In this study, electrospray is being used to investigate the reaction kinetics of lipid hydrolysis (Figure 6.6), along with selected ion monitoring. Initial electrospray experiments verified the identity of the observed ions as internal standard, product, and substrate. Selected ion monitoring was used to perform the calibration studies in which the product and internal standard were monitored simultaneously (Figure 6.6). Selected ion monitoring also allowed for the optimization of the experimental conditions. The electrospray kinetic measurements are currently under way.

Electrospray is a viable method for monitoring reactions that might otherwise require derivatization of the substrate. Electrospray ionization



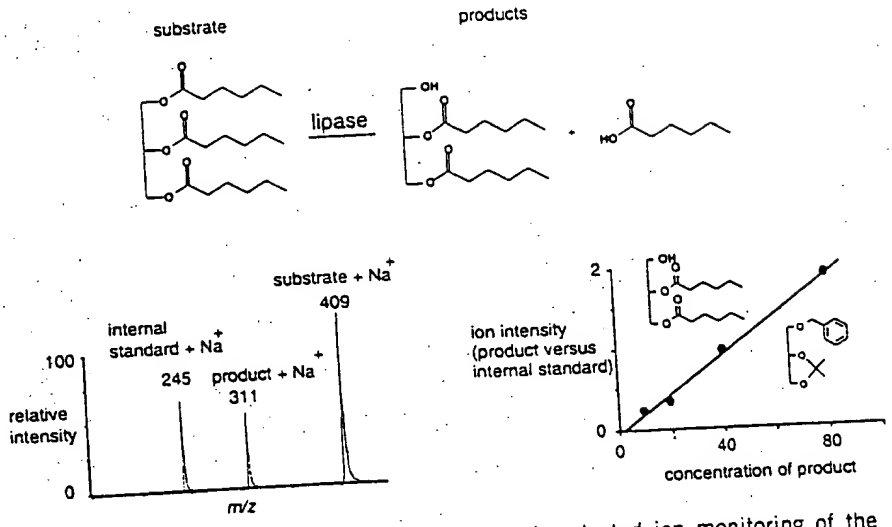


Figure 6.6 Reaction catalyzed by lipase (above), selected ion monitoring of the internal standard, product, and substrate (left), and calibration curve for relative product ion formation versus the product concentration (right).

mass spectrometry has also demonstrated its potential in the analysis of noncovalent interactions between an antibody and a hapten, and for observing covalent protein-bound intermediates in an antibody-catalyzed reaction.

References

Siuzdak, G., Krebs, J. F., Benkovic, S. J., and Dyson, H. J. (1994). *Binding of a hapten to a single-chain catalytic antibody demonstrated by electrospray mass spectrometry. J. Am. Chem. Soc.* **116**, 7937–7938.

Krebs, J. F., Siuzdak, G., Dyson, H. J., Stewart, J. D., and Benkovic, S. J. (1995). *Detection of a catalytic antibody species acylated at the active site by electrospray mass spectrometry. Biochemistry* **34**, 720–723.

Chavez, R. G., Bothner, B., Strupp, C., Hilvert, D., Siuzdak, G. (manuscript in preparation).

Protein Conformational Changes Using Deuterium Exchange and Electrospray Mass Spectrometry

Electrospray mass spectrometry has also been used to monitor protein folding. It was recognized that some proteins exhibit a distinct difference in the electrospray charge state distribution, dependent on their solution conformers. For example, two charge state distributions are shown in Figure 6.7 for a protein's native (less charged) and the denatured form (more

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(71) Applicant: PERSEPTIVE BIOSYSTEMS, INC. [US/US]; 500 Old Connecticut Path, Framingham, MA 01701 (US).

(72) Inventors: JINDAL, Satish; 20 Norman Street, Milton, MA 02186 (US). REGNIER, Fred, E.; 1219 Tuckahoe Lane, West Lafayette, IN 47906 (US). WILLIAMS, Kevin; 76 Beaconfield Road, Brookline, MA 02146 (US). AFEYAN, Noubar, B.; 6 Fairfield Drive, Lexington, MA 02173 (US). PALIWAL, Sandeep; Apartment 19, 1555 West Middlefield Road, Mountain View, CA 94043 (US). EVANS, David; 77 Malden Street, Natick, MA 01760 (US). PINGALI, Aruna; 1704 Windsor Ridge Drive, Westboro, MA 01581 (US).

(74) Agent: TURANO, Thomas, A.; Testa, Hurwitz & Thibeault, L.L.P., High Street Tower, 125 High Street, Boston, MA 02110 (US).

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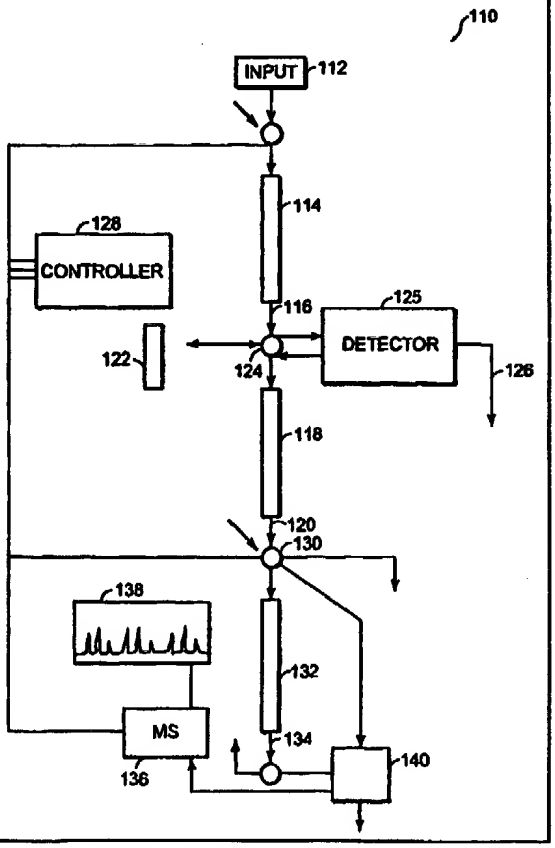
(57) Abstract

The invention provides novel methods for screening a sample to select a ligand to a target of interest and for obtaining information about the ligand and its binding characteristics. Specifically, the claimed multi-dimensional methods involve combining a solution of heterogeneous ligands with the target of interest to screen the ligands on the basis of one or more binding characteristics. Ligands having the first binding characteristic bind to the target of interest thereby to form a target/ligand complex. The complex then optionally is separated from the unbound components using any of a variety of separation techniques, e.g., size exclusion. At least one of the complex or unbound components then is introduced to a second "dimension". The second dimension is capable of separating components based upon a second binding characteristic. One then elutes the ligand having the desired binding characteristics.



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CZ	Czech Republic	LV	Latvia	TG	Togo
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EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
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HIGH SPEED, AUTOMATED, CONTINUOUS FLOW, MULTI-DIMENSIONAL MOLECULAR SELECTION AND ANALYSIS

RELATED APPLICATION

This application is based on provisional application serial number 60/000,518 filed June 26, 1995, the disclosure of which in its entirety is herein incorporated by reference.

FIELD OF THE INVENTION

This invention relates to the integrated, multi-dimensional, rapid analysis of solutions of a large number of mixed molecular species, commonly called "libraries". More specifically, the invention is directed toward methods for the discovery of molecular entities useful in a variety of biological contexts using hi-flux screening of natural and synthetic libraries to select ligands having a desired affinity for a target molecule of interest.

BACKGROUND

Multi-dimensional systems, i.e., systems involving the application of multiple distinct physico-chemical separation steps, are known to be useful for many applications. The purification of proteins, for example, frequently is accomplished using multiple passes through different chromatographic columns exploiting differential partitioning such as adsorption and size exclusion. Inherent in any multi-dimensional process is the necessity to identify the desired component, from the output of the first partition, to collect it, and to introduce it into the next dimension of the system. Disadvantages of such systems include slow analysis, solvent incompatibility between successive partitioning phases, the necessity of labor intensive handling, and consequent contamination or loss of sample.

The recent prior art discloses various new methods for implementing the search for novel agents such as, for example, pharmacological or therapeutic agents (i.e., drug discovery) agents useful in animal care or management, agriculturally useful chemicals, selective biocides for insects, weeds, or other pests, and catalytic and other entities useful in industrial processes. Collections of molecules or "libraries" are prepared and screened for molecules having a specified bioactivity, as

indicated initially by detection of binding between one or more species or "ligands" in the library and a "target" molecule with which it reacts to influence some biological process. More specifically, libraries consist of a complex assortment of molecules containing one or more ligands which may bind to a target of interest. The identification of ligands which bind may provide a lead for identifying compounds with a desired biological activity, e.g., as a potential drug candidate. As methods have become available to screen these complex mixtures more effectively, interest in exploiting this new "rational design" or "directed molecular evolution" approach has increased.

Libraries of biopolymers may be prepared by the sequential synthesis based on randomized addition of amino acid, nucleotide, or sugar residues, or combinations thereof, to form peptides, RNAs, polysaccharides, glycosaminoglycans or the like, thereby to prepare a random mixture of oligomers. Techniques suitable for preparing protein or peptide libraries at the nucleic acid level by phage display and similar technologies also are known. Likewise, these general synthesis approaches could be adapted to prepare, peptide nucleic acid (PNA) libraries, or libraries of PNA/DNA or PNA/RNA chimeras, and indeed other complex mixtures of synthetic molecules.

Screening of soluble peptide libraries frequently is performed either by immunoassay or by laboriously assaying for a particular biological function (e.g. blocking of viral replication). These methods are not necessarily target based and in most cases involve tedious set up. See Scott and Craig, Curr. Opin. Biotech. 5, 40-48 (1994); Dooley et. al. *Proc. Natl. Acad. Sci., 90:*10811-10815 (1993); Dooley *et. al., Life Sciences, 92*:1509-1517 (1990); Houghton *et. al., Biorg. Med. Chem. Lett., 3*:405-412 (1993). For example, inhibitors of HIV protease have been identified by screening sets of equimolar peptide mixtures, together containing more than 240,000 soluble tetrapeptides. See Owens *et. al. Biochem. Biophys. Res. Comm., 181:*402-408 (1991). It has also been suggested to use a phosphopeptide library to determine the sequence specificity of the peptide-binding sites of SH2 domains by employing the GST-SH2 fusion protein immobilized onto a column. See Songyang et al. Cell, 72, 767-778 (1993).

The screening methods described immediately above are based upon identifying which ligand in a mixture binds to a target of interest. Binding typically is assayed with either the ligands of the library or the target immobilized on some form of solid support. Various solution parameters may be adjusted to emulate different binding conditions and to obtain different ligands. Often, peptides which are obtained through procedures involving their immobilization to a support have disappointing affinity, i.e., have a binding constant too low to be useful. Traditionally, antibodies are used for the affinity purification of proteins and other biomolecules. However, the cost of generating antibodies, the potential for antibody leaching, and the need for relatively harsh eluting conditions pose problems for the routine use of antibodies in affinity purification.

Screening methods known in the art thus are not entirely satisfactory. Prior methods for detecting or identifying ligands which bind to a target of interest often fail to provide ligands of sufficiently high affinity to be useful, and additionally suffer from the loss of sample, the need for large amounts of ligands, and the need to vary loading, binding, or elution conditions to obtain useful results. Additionally, existing systems are unable selectively to screen a library while simultaneously determining the affinity of selected ligand(s) for the target under relevant conditions.

A major hurdle in the exploitation of current screening techniques of the type described above is effective chemical characterization of ligands identified in these processes. Chemical characterization, e.g., determining the sequence of an identified biopolymer, is at best time-consuming and complex. A major focus of prior art screening techniques is to enable the collection of enough of or enough information about a ligand of interest so as to permit determination of its structure and to enable synthesis of larger amounts for testing and further empirical structural refinement.

Accordingly, there is a need for integrated, multi-dimensional screening, selection and analysis systems and methods which permit automated, direct transfer of samples without dilution or loss between various dimensions, and efficiently screen for, and subsequently permit

characterization and recovery of ligands to a target of interest, even when present at low concentration.

SUMMARY OF THE INVENTION

Accordingly, the present invention is directed to rapid, efficient and automated, multi-dimensional systems, methods and apparatus for screening libraries to select, recover and characterize a candidate ligand with a desired or preselected affinity K for a preselected target molecule. Additionally, the present invention is directed to certain combination of individual dimensions of such a system, which can be used to obtain a desired result, and, specifically to a method of detecting a ligand to a target of interest which overcomes the disadvantages of the methods known in the art.

Additional features and advantages of the invention will be set forth in the description which follows, and in part will be apparent from the description and drawing, or may be learned by practice of the invention. The objectives and other advantages of the invention will be realized and attained by the process particularly pointed out in the written description, drawing, and appended claims.

To achieve these and other advantages, and in accordance with the invention as embodied and broadly described, the invention provides novel methods for screening a sample to select a ligand to a target of interest and for obtaining information about the ligand and its binding characteristics. Specifically, the claimed multi-dimensional methods involve combining a solution of heterogeneous ligands with the target of interest to screen the ligands on the basis of one or more binding characteristics. Ligands having the first binding characteristic will bind to the target of interest to form a target/ligand complex. The complex then optionally is separated from the unbound components using any of a variety of separation techniques, e.g., size exclusion. At least one of the complex or unbound components then is introduced to a second "dimension". The second dimension is capable of separating components based upon a second binding characteristic. One then elutes the ligand having the desired binding characteristics.

Additionally, the invention relates to a method of detecting the presence of a ligand having a desired or preselected affinity (K) for a preselected target molecule in a sample of ligands in a solvent by loading a column with a known concentration of target molecules (T), and passing the sample through the column so that ligands in the sample bind to the column through the target

molecule. A series (n) of column volumes of solvent then are passed through the column, where n is a number of column volumes between 1 and 10,000. A subset of the column volumes exiting the column is passed through a ligand accumulator to immobilize on the accumulator ligands having the preselected affinity K. Ligands having the preselected affinity then can be eluted from the accumulator, and, optionally, identified, and/or synthesized in commercial quantities.

In another aspect, the invention relates to a method of separating mixed species of ligand dissolved in a solvent into separate fractions of ligands, wherein each fraction is characterized by a different affinity or range of affinities for a preselected target molecule. Initially, the mixed ligand species are passed through a column comprising immobilized target molecules so that the ligands will bind to the target. A series of column volumes of solvent then are passed through the column, and at least two subsets of the column volumes of solvent exiting the column are then passed through a ligand accumulator, thereby immobilizing ligands characterized by separate ranges of affinity constants. The fractions containing ligands characterized by different ranges of affinities are then optionally eluted from the accumulator to separate them chemically for further screening or analysis.

In yet another embodiment, the invention relates to a multi-dimensional system or apparatus for obtaining and identifying ligands having a preselected affinity for a target of interest. The multi-dimensional system consists of at least two dimensions, the first comprises a chromatographic element to which a concentration, preferably a known concentration T, of target molecules of interest is bound. The system has as a second dimension another chromatographic element followed by a detector. Additionally the system in some embodiments has an interface between each dimension, and a controller for automatically regulating the various dimensions of the system.

In yet other embodiments, the apparatus comprises multiple valves, a first column with target molecules immobilized thereon; an accumulator or separate column to receive at least a portion of the exist stream of the first column, an optional interface to condition the exit stream to make it compatible with the accumulator or second column, and a detector such as a mass spectrometer. The interface may, in some embodiments include a buffer exchange such as a

mixed bed ion exchanger, a cation exchanger or an anion exchanger, and means to inject solvent so that the pH, ionic strength, etc. can be controlled so as to permit further downstream partitioning of partly screened ligand species.

The ability of the methods and apparatus of the invention to provide for continuous flow through multiple partitioning dimensions is dependent in many cases on the use of interface columns. These condition the solvent containing the dissolved ligands exiting an upstream column for effective partitioning in a downstream column. In one such interface, effluent high in salt is desalted by passage through a reverse phase column. The ligands adsorb, the salt is washed out, and the ligands then are eluted with salt-free or low salt solvent. In another, organic solvent such as acetonitrile is removed by passing the solution through an ion exchange column, binding the ligands therein, and subsequently eluting with an aqueous eluant. In still another, the pH of acidic solvents is increased by binding in a cation exchange resin, washing out the acid, and eluting in, e.g., a neutral pH solvent. Similarly, the pH of alkaline solvents may be decreased by binding in an anion exchange resin.

In yet other embodiments, the invention features an interface for sampling a liquid chromatographic (LC) exit stream, and delivering the sample to a mass spectrometer (MS). The sampler has a predetermined sample volume disposed, for example, in a sample loop, alternatively switchable to extract from an LC exit stream, and to insert into an analysis stream of an MS. A sample controller cycles the sampler to first extract and then to insert the sample. In various embodiments, the sampler can comprise a multi-port valve and the sample volume is disposed within tubing of a predetermined volume. The sample controller may cycle the sampler to take a sample of the LC eluate a plurality of times during an LC analysis peak. Other embodiments may include a second sampler. The first and second samplers can be placed in series.

The embodiments of the methods, apparatus and system of the invention described above may optionally include a detector for identifying a selected ligand. The detector may consist of, for example, a mass spectrometer or a fluorescence detector.

Additionally, in other embodiments, the invention relates to a method of detecting a ligand having a desired high affinity K for a preselected target molecule when the ligand and the target

are present together in preselected solvent conditions. The ligand to be detected may be one of a multiplicity of ligand species in a heterogeneous sample. The method involves immobilizing the target molecule onto a column, passing the sample through the column to promote binding of ligands in the sample to the target molecules, and then passing a series of column volumes of a solvent defining the solvent conditions. A subset (kp) of the column volumes exiting the column are then passed through a ligand accumulator to immobilize thereon ligands having the desired affinity, and then those ligands are eluted. A selected ligand may be characterized by a high affinity K for the target molecule equal to approximately (kp)/T, where T is the concentration of target molecules in the column. In some instances, the conditions under which the sample is passed through the column (i.e. to promote binding of ligands to target molecules) is different from the preselected solvent conditions.

In other embodiments, the methods of the invention relate to the detection of a ligand having a high on-rate, Ko, when said ligand and said target molecule are present together in preselected solvent conditions. The ligand is detected in a sample comprising multiple ligand species, at least one of which binds a preselected target molecule with an affinity of at least about 10^4M^{-1}. The target molecule is immobilized onto a column, and the sample is provided in a sample solution defining the preselected solvent conditions. The sample is passed through the column at a high linear fluid velocity so as to minimize the residence time of the ligands in the column, thus selectively binding high on-rate ligands to the target molecules, in preference to other ligands in the sample. One can then elute the column to obtain an output, and identify the high on-rate ligands. Optionally, the output can be passed through a ligand accumulator, which is then eluted to produce an output rich in a high on-rate ligand.

In other embodiments, the invention relates to a method of selecting ligands to a target of interest on the basis of the off-rate of the ligand.

The methods of the invention in certain aspects relate also to a method for detecting a ligand having a high affinity for a target molecule by providing a library obtained by the digestion of one or more proteins or other biopolymers. The sample solution and a target molecule are combined under conditions which allow suitable ligands, if present, to bind to the target; and

thereafter the ligands which bind to the target (forming a complex) are separated from those which do not bind. The sample solutions may be obtained by the digestion of any protein, including post-translationally modified proteins, antibodies, etc.

The methods of the claimed invention also relate to detecting a ligand in a library which will bind when the ligand and the target molecule are present together in preselected solvent conditions, e.g., physiological saline. As before, a target molecule is immobilized onto a column, and the sample is passed through the column under the preselected solvent conditions. Next, a series of column volumes of solvent is passed through the column to select a desired ligand. The eluate is then introduced to a ligand accumulator.

The methods also relate to the preparation of pharmaceutically active compositions using the multi-dimensional methods described above, and to the subsequent commercial production of such compositions.

In yet other aspects, the invention relates to methods of selecting ligands based upon one or more binding characteristics by the use of multiple dimensions.

In an important aspect, the invention provides apparatus and methods which are automated, fast, and operate by continuous flow. The methods are capable in preferred embodiments of selecting ligands having affinity and specificity for essentially any target molecule, separating the members of the select group from one another, and obtaining physico-chemical data characteristic of the structure of the selected ligands. The nature of the library useful in the system essentially is unlimited. Thus, mixtures of organic compounds may be used. Digests of biopolymers, either natural or synthetic, are particularly attractive. Such digests may comprise mixtures of peptides, polysaccharides, polynucleotides, various derivatized forms thereof, and variously sized fragments thereof. The biopolymers may be extracted from plant or animal tissues, diseased or healthy, digested if necessary, or used as is. Such libraries are available in abundance, easy to prepare, may be of lower toxicity and more stable than synthetic peptides, and may be varied and screened systematically.

It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory and are intended to provide further explanation of the invention as claimed.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a schematic representation of a splitter interface (sampler) between a liquid chromatography column and a mass spectrometer in the apparatus of the invention (Position A).

Fig. 2 is a schematic representation of a splitter interface (sampler) between a liquid chromatography column and a mass spectrometer in the apparatus of the invention (Position B).

Fig. 3 is a schematic representation of one embodiment of the apparatus of the invention.

Fig. 4 is a schematic representation of a second embodiment of the apparatus of the invention.

Fig. 5 is a diagram of a BIOCAD™ Workstation available from PerSeptive Biosystems, Inc. which is plumbed in tandem column mode. Column 1 is a weak anion exchange column and column 2 is a reversed phase column.

Fig. 6 depicts target-based screening of human rHsp70.

Figs. 7A, 7B, 7C and 7D depict MALDI spectra of natural peptide library screen vs. rHsp70 and Dnak. Fig. 7A depicts the MALDI spectrum of a rHsp70 sample incubated with the PDL. Fig. 7B depicts the MALDI spectrum of a control incubation with rHsp70 alone. Fig. 7C depicts the MALDI spectrum of a control incubation with PDL alone. Fig. 7D depicts the MALDI spectrum of Dnak incubated with PDL.

Fig. 8 identifies peptide sequences binding to Concanavalin A in a sugar-specific manner.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

Accumulator - a column designed to non-specifically adsorb ligands and optionally and preferably to permit the separation of the adsorbed ligands by elution.

Affinity Column - a column containing immobilized target molecules accessible to ligands passing therethrough so as to permit formation of a complex.

Biopolymer - a polymeric molecule of biological origin comprising plural attached monomers, derivatized or not, including amino acids, DNAs, RNAs, PNAs, sugar residues, or combinations thereof.

Column - used broadly herein to refer to laboratory scale or microscale chromatography columns, i.e., pipes packed with porous or nonporous, rigid or gel particles, or containing one-piece matrices, designed for chromatographic separation, or functional equivalents of such columns including membranes and capillaries or bundles of capillaries.

Complex - a non-covalent association of a candidate ligand and a target molecule.

Continuous Flow - systems wherein impelled solutions containing ligands impelled by a pump are passed sequentially through various columns, valves, detectors, interface units, etc. within the system without requiring collection or separate analysis manually or robotically until the solution exits the system.

Eluate - the fraction of liquid exiting a column containing solutes that were sorbed on the column and then desorbed by various types of elution, including gradient elution and isocratic elution techniques, and elution by changing pH, ionic strength, or other parameters of solutions within the column, or simply by passing a large volume of buffer through the column.

Exit Stream - a collective term referring to either the portion of liquid exiting a column containing solutes which failed to bind to the column or an eluate.

Library - a collection of structurally distinct ligand species comprising intact or fragmented organic molecules or molecules of biological origin such as peptides, nucleotides, polysaccharides, and various derivatized forms thereof.

Ligand - a generic term referring to the structurally distinct chemical species that are dissolved in a solvent and constitute a library.

Mass spectrometer - a machine adapted for the introduction of microsamples containing a selected ligand or fragments of a ligand, or which generates ligand fragments, and measures the mass to charge ratio of solutes in the sample to provide data helpful or sufficient to determine the structure of the ligand.

MS/MS - a mass spectrometer detector of the type that determines the mass to charge ratio both of a ligand inserted therein and then of fragments of that ligand generated by ionization or otherwise.

Reverse Phase - a chromatography surface characterized by an abundance of hydrophobic moieties.

Target Molecule - a compound such as a receptor, enzyme, DNA, RNA, etc. comprising either a) the moiety to which a selected ligand will bind with at least some selectively and reasonably high affinity, i.e., is the molecule which will be exploited during use of the selected ligand, or b) is a moiety which the selected ligand is selected specifically not to bind, so as to avoid cross reactivity or the like.

I. NATURE OF LIBRARY

A "library" as used herein encompasses virtually any solution of compounds to be screened for a ligand having an activity of interest. The library may, for example, comprise a natural or synthetic combinatorial library; solutions obtained naturally, such as from body fluids, plant fluids; or virtually any other natural or synthetic substances which can be put into solution and detected by a physical or chemical characteristic. Thus there are limitless sample possibilities and the skilled artisan can choose a sample based upon his particular application.

In various embodiments it is preferable to use a natural library of molecules obtained by the digestion of one or more natural substances. When screening for lead compounds for pharmaceutical applications (i.e. drug discovery), the sample may be obtained by the digestion of one or more molecules obtained from the host organism. It may be preferable to digest a molecule indigenous to the host which has the desired biological activity. The inventors have discovered that these libraries have a high likelihood of containing a fragment having the desired activity against a target of interest.

Natural libraries may be prepared by enzymatic digestion or other manipulation of a sample prior to screening, or in certain instances, may be a solution as found in nature without

prior manipulation.. The use of proteolytic enzymes to generate peptides of pre-existing amino acid sequences through simple degradation of existing proteins is well known. (U.S. Patent No. 3,855,196; Pieczenik, WO87/01374; U.S. Patent No. 5, 366,862) Preferably one may digest the target molecule or a related molecule to create a library of potential ligands related to the target peptide. Thus, if one desired a ligand which binds with a preselected affinity to a growth hormone, a library of potential ligands could be prepared by digesting a known ligand to the growth hormone, and then screening for the desired ligand. For example, one may obtain proteolytic digests of mixtures of common proteins that are commercially available from a company such as SIGMA. These mixtures are referred to as either natural tryptic peptide mixtures or natural tryptic/chymotryptic peptide mixtures. Similarly, one may, for example, raise polyclonal antisera to a preselected target molecule, and digest the immunoglobulin in the sera to produce a mixture of candidate ligands.

Additionally the invention contemplates the use of natural libraries which have not been subjected to digestion prior to screening. Thus, one may obtain a sample by extracting animal or plant cells, tissue or fluids such as body fluids such as saliva, semen, vaginal fluids, and blood, as well as a naturally occurring libraries such as a cellular lysate or fermentation broths. Such libraries may optionally be manipulated by various processes prior to screening.

Such naturally obtained libraries are advantageous for a number of reasons. First, the likelihood of identifying a ligand which will be toxic to a host is diminished if the library is created by enzymatic digestion using typsin or chymotrypsin, for example, as these enzymes are found in the human body and are thus unlikely to have toxic effects. Second, since, for example, many proteins are post-translationally modified, one can obtain fragments having these modifications. This is especially beneficial in applications where the modification is involved in the biological activity of the molecule or fragment. Such post translationally modified proteins may include, for example proteins which are sulfated, amidated, carboxylated, phosphorylated, disulfide bonded, or lipidated.

II. THE MULTIDIMENSIONAL APPROACH

A. THEORETICAL BASIS FOR SCREENING

Peptide combinatorial libraries and natural proteolytic mixtures contain three types of peptides; those that i) have no affinity to any protein, ii) bind to a large number of proteins, or iii) show affinity to a specific protein. The later group may be further subdivided according to binding affinity and the specific site on the protein surface to which the peptide binds. It is necessary in a "screening" system to differentiate between these various peptides.

It has been noted above that protein also referred to herein as the target (R)/ligand (L) association may be described by the formula

$$[R] + [L] \text{ ----->} [RL] \qquad (A)$$

and the equation

$$K_b = k_1/k_2 = [RL] / [R] [L] \qquad (1)$$

where K_b is the binding constant and the rate constants k_1 and k_2 represent the forward and reverse rate constants, respectively. The general way in which peptides from synthetic libraries are screened is i) to use an excess of peptide, ii) control the conditions of association, iii) allow the system to come to equilibrium, and iv) then rapidly separate the unbound peptides from the RL complex. From this point on the various screening systems diverge in the identification of bound peptides.

The claimed approach is quite different than that used by others. The methods described herein allow us to select on the basis of the forward rate constant of a ligand for the receptor, the reverse (off) rate constant, or the equilibrium constant under conditions where it is possible to vary ionic strength, pH, concentration of competitive binding agents, organic solvent concentration, and temperature to name a few. All of these conditions potentially impact complex (RL) formation.

Selection of peptides based on their binding constant can be achieved in several different ways. One is through the use of a chromatography column with the receptor (R) immobilized. Another is in a chromatographic system in which the components of the RL complex are separated as the complex dissociates.

In the immobilized receptor approach, the receptor (R) is immobilized on a chromatography column. Since the association of ligand with immobilized receptor is biospecific, the affinity of that association is dictated by the equilibrium as show in eqn. 1 above. The equilibrium constant (K_b) may be related to the chromatographic behavior (k') by the equation

$$K_b [R] = [RL] / [L] = K_d = k' / \theta \quad (2)$$

Where K_d is the chromatographic distribution coefficient and θ is the phase ratio. Rearranging the equations above it may be shown that

$$k' = K_b [R] / \theta \quad (3)$$

When receptor concentration [R] is large relative to [RL], then [R] may be assumed to be constant. Because the phase ratio (θ) is also a constant, k' in the isocratic elution mode is directly proportional to the binding constant K_b.

The impact of band spreading on the screening process must also be considered. Estimation of band spreading is generally related to theoretical plates (N) in which

$$N = 16 (V_e / v)^2 \quad (4)$$

where V_e is the elution volume of the analyte either in ml/min or column volumes (CV) and v is the peak width in the same volume units. At medium to high mobile phase velocity it is probable that columns will have 100 plates or less and plate heights will be 2 mm or more. This would mean that

$$V_e = 2.5 v \quad (5)$$

It may be concluded from these equations that peaks will be very broad and it will be difficult to determine the peak maximum, i.e. k'. Furthermore, the resolution as defined by the equation

$$R_s = (V_{e2} - V_{e1})/v \quad (6)$$

shows that when $v = 0.4 V_{e2}$, it is equivalent to a 100 plate column.

Thus, when peaks are very broad, detection sensitivity is seriously compromised and it is difficult to determine k' as noted above. One solution is to collect and concentrate fractions of ligand eluting from the affinity column over a fixed time period and determine ligand

concentration in the accumulated fraction. This may be achieved by using a reversed-phase chromatography column as an accumulator by coupling it in tandem with the affinity column. To determine k' by this method, multiple samples must be collected and quantitated to allow reconstruction of the chromatography peak. Assuming that the peak will always be of the same shape, k' may be estimated by determining the peak width and the fractional amount of the analyte eluted at any point in time.

The chromatography column is essentially one theoretical plate, i.e. 1-10 mm length, which is saturated with ligand (L) to form RL complex. Although substantial quantities of RL may be formed in the loading process, it is still possible that there is a finite quantity of residual receptor (R), especially as ligand elutes from the column. Elution of ligand (L) from this column depends on the dissociation process (formula B) in which

$$[RL] \text{ ------- } > [R] + [L] \qquad (B)$$

Free ligand is swept from the system before it has the chance to recomplex with R to form RL. Because the binding constant is very large, i.e. $> 10^6$, most of the ligand exists in the column in RL complex. This means that the rate of elution of ligand from the column will be described by the equation

$$d[RL] / dt = - F[L] / V_c \qquad (7)$$

where F is the volumetric flow rate (ml/min), [L] is ligand concentration, and V_c is the column volume (ml). Integration indicates that

$$\log [RL] = \{[L]F / V_c\}\log 1/t \qquad (8)$$

But we know that

$$K_b = \{[RL]_i - [L]\} / \{[R]_i + [L]\}[L] \qquad (9)$$

where K_b is the binding constant, $[RL]_i$ is the initial concentration of the RL complex, and $[R]_i$ is the initial concentration of free receptor. Substituting for [L] in the integrated form of the equation allows one to predict the rate of elution of ligand from the column as a function of the binding constant.

One may computer model the system in an iterative process in which a new equilibrium is computed each time one column volume saturated with ligand is swept from the column. In so doing it is assumed that the system is in equilibrium at all times and there are no significant mass transfer limitations.

Using binding constants that vary by orders of magnitude it can be shown that there is a strong selection for species with large binding constants to stay bound to the column while species with smaller binding constants are eluted.

When i) a mixture of ligands (L_1, L_2, L_3 ---------- L_n) having binding constants for a receptor (R) exceeding 10^6 are brought in contact with immobilized (R) and ii) the sum of the concentration of these ligands exceeds that of the receptor, the receptor will be saturated. Furthermore, when this system is allowed to come to equilibrium the relative concentration of the various species will be represented by the equation

$$Kb_1[L_1] / [RL_1] = Kb_2[L_2] / [RL_2] = Kb_3[L_3] / [RL_3] = Kb_n[L_n] / [RL_n] \qquad (9a)$$

Because the initial concentration of the various ligands is unknown, it is not possible to estimate binding constants on either a relative or absolute basis.

This is known as the equilibrium shift method. The equilibrium shift method is based on the following protocol. First an immobilized receptor column located on one of the valves of a multivalved liquid chromatograph is saturated with ligand(s) and the adsorbed ligands subsequently desorb from the immobilized ligand column and reconcentrate on a reversed phase column through valve switching that couples the receptor and reversed phase columns in tandem. The receptor column is then switched out of the system and the ligands are separated by gradient elution from the reversed phase column and quantitated.

Second, the immobilized receptor is again saturated with the ligand mixture. Free ligands are then rapidly eluted from the system with a 1.5 column volume wash that is discarded to waste. The receptor column is then switched into a fluidic loop in which liquid is pumped from a reservoir of volume (V') through a high pressure pump into the receptor column and then back into the reservoir. The volume of the pump, connecting tubing, and receptor column is V''. The liquid volume (V) of the system is the sum of V' + V''. Receptor:ligand complex [RL] will dissociate until the system once again comes to equilibrium as described by equation 1 above.

Let us consider a chromatography column of surface area As, saturated with ligand in which receptor density is [R], the density of complex (RL) is equal to [R] = [RL], and the amount

of ligand adsorbed to the column is $[RL]_i As$. In the special case where 50% of the ligand initially adsorbed on the column desorbs from the surface and enters the liquid phase, i.e.

$$[RL]\ As = [L]V = 1/2[RL]_i As = [R]As \tag{10}$$

then

$$K_b[L]V = 1 \tag{11}$$

In the more general case

$$K_b\{a / (1-a)\}[L]V = 1 = (K_b\{a / (1-a)\}[RL]V) / 2 \tag{12}$$

where a is the fraction of the initial adsorbed ligand that dissociates and enters the liquid phase and (1-a) is the fraction of the initial RL complex remaining after reequilibration. When two substances are bound

$$K_{b1}\{a_1 / (1-a_1)\}[RL_1]V = 1 = K_{b2}\{a_2 / (1-a_2)\}[RL_2]V = 1 \tag{13}$$

to the column and K_{b2} is the known, the equation

$$K_{b1} = K_{b2}\{a_2 / (1-a_2)\}[RL_2]\{(1-a_1) / a_1\}(1/[RL_1]) \tag{14}$$

allows one to calculate the binding constant K_{b1} based on the relative amounts of the two substances eluted from the column when the equilibrium shifts to compensate for the increase in volume (V).

As noted above, screening may also be achieved in a chromatographic system by chromatographing the RL complex and separating receptor (R) from ligand (L) as the complex RL dissociates to prevent reassociation. In this process, RL complex of those ligands with the highest binding affinity will be the most likely to survive passage through the chromatographic system without dissociation. The rate at which R is separated from L, i.e. resolution (R_s) as a function of time (dR_s / dt), is an important issue. Resolution in a chromatographic system is shown in eqn. 6, where $R_s = (V_{e2} - V_{e1}) / v$. Because dt is inversely related to mobile phase velocity (V_m),

$$dR_s / dt = V_m(V_{e2} - V_{e1}) / v \tag{15}$$

When elution volumes are converted to capacity factor and peak width expressed in terms of plate height and column length, equation 15 becomes

$$dR_v/dt = [(k'_2 - k'_1)V_m(LH)^{1/2}] / 4(k'_2+1) \quad (16)$$

In the case of size exclusion chromatography (SEC), when both R and RL are excluded from the pores, $k'_1 = 0$ and eqn. 16 reduces to

$$dR_v/dt = [k'_2V_m(LH)^{1/2}] / 4(k'_2+1) \quad (17)$$

Because peptides are small they will generally elute from an SEC column in the totally included volume, i.e. $V_0 + V_i$. This means that

$$K'_2 = V_i / V_0 \quad (18)$$

In this special case eqn. 18 becomes

$$dR_v / dt = [(V_i / V_0)V_m(LH)^{1/2}] / [4\{(V_i / V_0)+1\}] \quad (19)$$

The case of size exclusion chromatography (SEC) is very similar to a dialysis system in which the inability of a macromolecular receptor to penetrate a pore matrix excludes it from certain liquid elements of the system. In the SEC system the RL complex dissociates and ligand (L) diffuses into the pores of the SEC matrix from which RL and R are excluded. Because the macromolecular R receptor moves through an SEC column faster than the low molecular weight ligand (L), R moves away from the zone of L in the pores of the support. This precludes reassociation to RL. When this separation of R and L has occurred, more RL must dissociate to maintain the equilibrium in eqn. 1. When this separation of R and L occurs very rapidly, maintaining the equilibrium will become dependent on the off-rate (k_2). This means that a low mobile phase velocity selection will depend on the equilibrium constant (K_b) while at high mobile phase velocity selection will be based on the rate constant k_2.

Screening based on the rate of dissociation of the RL complex may be achieved in several ways as described above. In the systems described above, the concentration of receptor [R] increases as ligand elutes from a section of the column. Because the rate of complex formation is much higher than the rate of dissociation, it is impossible to carry out off-rate selection in porous chromatography sorbents. The concept described below allows off-rate selection by exploiting

the fact that subsequent to complex dissociation there is a low probability that ligand will contact a receptor bearing surface again before elution from the system.

Mass transfer to the walls of open tubular columns eluted with aqueous mobile phases is known to be poor. Mass transfer to the walls of open tubular columns decreases with the inverse square of increasing column diameter and the square of increasing linear velocity of the mobile phase. Columns of 300 -1000 μm with immobilized receptor (R) are loaded by filling the column with a series of ligands and allowing the system to come to equilibrium at zero mobile phase velocity. When the sum of the concentration of ligand species ($[L_1]$ --- $[L_n]$) > [R], there is competition between the ligands for a binding site. The amount of any RL complex formed is a function of both the constant of the particular ligand for the receptor and the concentration of that ligand. After equilibrium has been achieved, unbound ligands are swept from the column. Elution of L in bound RL complex is based on the off-rate. Assuming that subsequent to dissociation of RL to L there is no reassociation, the rate of ligand (L) elution from the column is given by the equation

$$-d[RL] / dt = k_2[RL] \quad (20)$$

where k_2 is the off-rate constant. Integrating between the limit of the initial concentration $[RL]_i$ of complex and the concentration [RL] at time t produces the equation

$$2.3 \log ([RL] / [RL]_i) = -k_2 t \quad (21)$$

The off -rate and the rate at which ligand are eluted from the column may also be expressed in terms of the half-life ($t_{1/2}$), i.e. the time it takes for half the ligand to elute from the column. From the equation above it may be shown that

$$t_{1/2} = 0.693 / k_2 \quad (22)$$

In summary, the basic assumption in this model of off-rate selection is that there will be no reassociation of R and L at the walls of the capillary once dissociation has occurred. This probably is not strictly true. The walls of a capillary are not well swept, i.e. there is a stagnant layer of liquid at the walls. L must diffuse through this layer before it escapes into the rapidly moving liquid in the center of the capillary where it is rapidly transported out of the capillary. The validity of using this model is that mass transfer i) dominates selection processes in

chromatography columns and ii) it is orders of magnitude poorer in large open tubular capillaries than porous particles.

A family of chromatography columns have been developed over the past decade based on the concept that liquid chromatographic separations may be achieved i) through the use of porous matrices in which access of analytes to the interior of a particle is controlled by molecular size and ii) the chromatographic stationary phase is only on the inside of the particle. These "restricted access media, have been particularly useful in the separation of low molecular weight drugs in serum from proteins. The first of these columns was the "internal surface reversed phase" (ISRP) media.

These columns, as they apply to screening, may be thought of in the following way. Because large receptor molecules would be excluded from access to the interior of ISRP media, they may be thought of as being similar to a dialysis membrane. In a dialysis system, the macromolecular receptor is restricted to one side of the membrane because it is excluded from passage through the pore network of the membrane by its physical size. In contrast, small ligands may penetrate the pores of the membrane and gain access to all the liquid space within the system. Dialysis is achieved by repeatedly removing liquid containing ligand from the non-protein containing side of the membrane.

The ISRP chromatography system functions in screening by i) allowing only ligand (L) to gain access to the interior of particles which contain stationary phase, ii) capturing L with high affinity on the internal reversed phase when the RL complex dissociates and L diffuses into the interior of the ISRP containing particle, and iii) transporting RL complex through the chromatography column. This system will be vastly superior to the dialysis system based on the fact that i) it actively captures the low molecular weight species (L) being removed from the protein and ii) the stripping process is repeated many more times in the chromatography column than is practical in a dialysis system.

Still another characteristic of the ISRP approach is that diffusion distances to the surface of the particle, where L is captured, are small and L will be quickly captured following dissociation from the RL complex. This means that the rate of removing L will be dependent on the rate of dissociation, not the equilibrium constant, K_b, and it will also be possible to select from mixtures of many RL species.

B. CONFIGURATIONS

In certain embodiments, the invention may comprise a first column having the target of interest immobilized thereon. Thus, when the sample is passed over the first column, ligands which bind to the target will be immobilized thereon by forming a target/ligand complex. The complex captured by the first column is dissociated by varying the wash volumes.

The claimed invention provides a method, system and apparatus for obtaining information about a particular ligand without the need for manual manipulations, regardless of the size of the sample, or the amount of ligand present in the sample. These methods remarkably provide for the first time, rapid, automatable means for drug discovery. Literally millions of compounds can be screened for a particular biological activity in a short period of time, thus leading to significant advances in the field of biotechnology, pharmaceutical development, diagnostics and therapeutics. The methods of the invention allow the artisan to select ligands to a target of interest based upon any one or more characteristics, such as (1) the forward rate constant of a ligand for the target, i.e. on rate, (2) the reverse rate constant, i.e., off-rate, or the equilibrium constant under conditions where it is possible to vary ionic strength, pH, concentration of competitive binding agents, organic solvent concentration, and temperature, for example. Any conditions which may potentially impact the formation of a target/ligand complex may be varied and used as a selection criteria. The methods of the invention also allow the rapid selection of a ligand to a target of interest, characterization of virtually any potential binding characteristics of the ligand, and recovery of the ligand.

The methods of the invention use a tandem column chromatographic technique: any column capable of separating molecules can be used in the methods of the invention. Thus, depending on the result sought, the columns in the system may be chosen from the group consisting of affinity columns, size exclusion columns, and/or reversed phase columns. As used herein, the term "tandem mode" indicates that at least two columns are involved in the system, either simultaneously, or sequentially. If the columns are run simultaneously in tandem mode, then sample solution may be split and delivered to each column. If they are run sequentially, the

columns are arranged so that the eluate of one column is directly introduced into the second column.

The claimed methods in various embodiments, may employ an affinity based column. In an affinity based screen, tandem-chromatographic columns are used to screen for ligands in a sample.

Reference will now be made in detail to the presently preferred embodiments of the invention. The multi-dimensional methods of the invention in various embodiments relate to detecting the presence of a ligand having an affinity K for a preselected target molecule in a sample of heterogeneous ligands dissolved in a solvent. The invention contemplates both the methods themselves, as well as multi-dimensional systems or methods including one or more dimensions. In various claims, the invention relates specifically to various dimension embodiments which are novel. The invention allows one to recover target-bound ligands, as well as simultaneously select ligands and determine their relative affinities during the screening process. As embodied herein, the invention relates to a multi-dimensional method, apparatus and system for the highly sensitive detection and analysis of ligands or analytes in samples, and to assays and other "dimensions" of the system. The invention comprises a multi-dimensional system which may include, for example, the screening of libraries, selection of ligands, and recovery and ligands with a desired activity, and subsequent identification. More specifically, dimensions may include any method of partitioning components of a sample, including, for example, immunoassays, such as affinity chromatography, reversed phase chromatography, and size exclusion chromatography. The techniques described in the specification and claims herein provide a multidimensional approach to screening samples, in which separations, chemical reactions, and mass spectrometry are integrated, and, preferably, automated.

The multi-dimensional system and methods may, in various embodiments include the following steps: (1) generation of a sample of potential ligands; (2) providing a support with a concentration of a target molecule immobilized thereon; (3) screening for a ligand to the target molecule, wherein the ligand has one or more desired properties; (4) separation of those ligands desired; (5) recovery of the ligand obtained; (6) identification of the selected ligands; and (7) large

scale synthesis of the ligand or derivatives thereof for diagnostic or therapeutic applications. The most effective combination of analysis dimensions can easily be determined by one skilled in the art based upon the particular results desired.

Such multidimensional systems allow the rapid screening of libraries of ligands for their ability to bind to a certain "target molecule" ("target"or "receptor") of interest. The target molecule can be any molecule to which a ligand is desired, such as, for example, proteins, peptides, nucleic acids, monoclonal or polyclonal antibodies, etc.

Screening soluble libraries of peptides or small molecules for identification of ligands binding to a specific target molecule has become a widely used technique in the pharmaceutical industry. Screening these libraries may lead to development of novel therapeutics and/or diagnostics. Screening requires both a selection process and a method to assess the relative affinities of the ligands. Methods known in the art thus require two separate steps to determine the acceptability of a particular ligand. Often, after screening the sample and recovering a desired ligand, it is discovered that the ligand selected has an undesirable affinity for the ligand. As will be recognized by one skilled in the art, different applications often require ligands having a specific affinity. Numerous variables must be taken into account to determine the applicability of the selected ligand for the desired application. For example, one may prefer a ligand having a particular on- or off-rate, or a ligand which binds to one target molecule, but not another. Until the present invention, therefore, the entire selection process involved several separate steps, in order to not only identify a ligand to a target, but to further select based upon additional characteristics.

The claimed methods allow the novel and rapid determination of relative affinities for ligands which bind to a selected target molecule, additionally, the methods provide for the recovery of the target-bound ligands, and offers the simultaneous selection of ligands and determination of their relative affinities or other binding characteristics during the screening of a library of compounds

Multi-dimensional analysis systems as disclosed and claimed herein provide multiple embodiments suitable for the screening, selection and recovery of desired ligands. It is preferable

to automate these assays, however, solvent compatibility has historically been a significant drawback to this approach. The inventors have avoided this problem by incorporating a universal solvent exchange method into their multi-dimensional assay systems.

In various embodiments, the invention relates to multi-dimensional separations which provide for the high resolution of complex mixtures. Because of the ability to directly couple various analytical components, sample handling and transfer steps can be virtually eliminated. This is especially critical when one is attempting to isolate and detect ligands present at very low detection levels.

The claimed system contemplates a multi-dimensional system which may incorporate a series of chromatography columns such as size-exclusion, ion exchange and reverse phase, as well as one or more detectors, such as a mass spectrometer.

The integrated coupling of various dimensions such as micro column affinity chromatography with capillary reverse phase HPLC/ electrospray ionization mass spectrometry in an automated multi-dimensional system should permit a highly sensitive and highly selective approach to decoding complex mixtures. The system should allow for rapid column and solvent switching capabilities. One skilled in the art can easily add extra dimensions to the system by subjecting the sample, or a subset thereof, to more separation or identification processes. Any suitable "dimension" may be added to the system, i.e. any separation processes such as two different chromatographic columns, or any other separation processes, that produce different spatial or temporal distributions of the individual components of the system.

The claimed invention encompasses a multi-dimensional system comprising two or more chromatographic or other separation dimensions which are plumbed in tandem mode. In a basic embodiment, two chromatography columns are plumbed in tandem mode. The first column is selected based upon the desired application and the basis of the sample. The first column may be an affinity chromatography, i.e. a column containing a stationary porous medium that has a different affinity for the various components in the sample; however, one may also use a non-porous medium. This column can be any solid support having immobilized thereon the target of interest. Such preselected targets may be molecules such as receptors, enzymes, nucleic acids,

polysaccharides, mucopolysaccharides, antibodies or binding proteins. Additionally useful target molecules include major histocompatibility molecules, T-cell receptors, antigens, cell-adhesion molecules, cellular receptors for hormones and growth regulatory factors and virus receptors. Ligands to these targets of interest provide a collection of possible immune agonists, antagonists, antiviral ligands and structural lead compounds for the design of small molecules with a desired bioactivity. As the sample solution passes through the support, ligands to the target will adsorb at the binding sites, and be retained on the column. A certain amount of solution components may also non-specifically bind to the support.

The affinity columns to be used in the invention may comprise any solid support which does not affect the binding activity of the target molecule. Supports commonly utilized are controlled pore glass, silica, silica gel, membranes, polystyrene based beaded supports, glass fibre frits and paper filters. While perfusive matrix materials are preferred, the invention also can be practiced with a non-porous matrix, in which tortuous channels are formed by the interstitial space among non-porous packed particles. These matrices have a lower net capacity than perfusive matrices but they may be very useful for microanalysis. In addition to packed particles, matrices useful in the process and apparatus of the invention may be embodied as bundles of microcapillaries. A high surface area/volume ratio may be provided by the use of very small internal diameter capillaries, providing a reaction vessel of a few microliters/cm. Likewise, the binding protein may be coated on the inner surface of the capillary tube. Solutes may be transported through the capillary tube matrix by convection. The high surface area to volume ratio of the capillary tubes increases the available reaction volume. The matrices may further comprise a membrane structure.

The matrix preferably is a rigid substantially non-microporous, particulate material having a hydrophilic surface, and preferably is also a perfusive chromatography matrix. The matrix also may be defined by the interior surface of a capillary. The methods comprise first loading a column with a known concentration T of target molecules.

In an alternative embodiment, the first column may comprise a size-exclusion column or dialysis system, capable of separating components of the sample based on size. The different

components of the sample will migrate through the column at different rates; ligands which have bound to the target, thereby forming a complex, will elute prior to the elution of smaller molecular species. Thus, the first portion of the exiting stream will contain the ligand bound to the target. It is preferable to use particles capable of perfusion chromatography since perfusion particles allow the system to operate at very high flow rates while maintaining both high sample loading capacity and chromatographic resolution. Preferably, the perfusion particles are POROS® beads available from PerSeptive Biosystems Inc. (Framingham, MA).

In various embodiments of the invention, a second column, plumbed in tandem mode with the first, is yet another chromatography separation column. Specifically, the second column may be an affinity column, as described above, having immobilized thereon a second target of interest having different physicochemical properties than the target immobilized on the first column. This configuration will enable one to select and separate ligands based upon one or more different physico-chemical properties. It may also be envisioned that both the first and second column have immobilized thereon the same target of interest, as a confirmation assay.

Alternatively, the second column may be an accumulator, i.e. a column designed to non-specifically adsorb ligands and optionally, to permit the separation of the adsorbed ligands by elution. A reversed phase column is a preferable accumulator.

Increasing the wash volumes will result in the highest affinity ligands being retained on the column for the longest period of time. Low affinity ligands, conversely, will have a shorter retention time on the column. There is a direct correlation between the wash volumes employed to wash ligands bound to the first column and their relative affinities.

In various embodiments, the wash volumes passed over the first column are indicative of the dissociation rate from the target. For example, if various ligands are passed over the first column and incubated to equilibrium, the presence of a particular ligand in the eluate must be a factor of the dissociation rate from the target. The dissociation rate is dependent on time, dilution, and association/dissociation rate constants. The time and dilution factors may be controlled, and thus, the loss of ligand bound to its target under these conditions should be directly related to the dissociation rate constant (i.e. off-rate) of the ligand. The target molecule

may be preequilibrated with a ligand prior to immobilization, or prior to introduction of the soluble library.

Thus, in certain embodiments, the first affinity column having a target immobilized thereon, is plumbed, in tandem mode, to an accumulator, such as a reversed phase column to immobilize thereon ligands having the desired affinity.

The elution of the reversed-phase column allows recovery of the ligands, and the peak heights of corresponding peaks provide a measurement of the amount of the ligand bound to the target at a specific wash volume. Under these conditions, the wash volume correlates with the dissociation rate constants of the ligands, and may provide an indication of relative affinities of the ligands having a similar mode of interaction with a target. The methods can be applied to the screening of a library of compounds where selection of ligands and determination of their relative affinities can be accomplished simultaneously, enabling selection of binders with the desired affinity.

Thus, for the first time, the practitioner is able to select a ligand based not only on its ability to bind to a target, but also upon its affinity for that target, i.e. the on-rate or dissociation rate (off-rate). This ability is especially relevant in drug screening applications where the dissociation rate may be critical to the effectiveness of the composition. Since the ligands bound to the target can be later recovered, further characterization or development of the selected binders can be performed, as discussed in more detail below.

The methods allow the practitioner to select ligands to the target of interest using virtually any affinity selection method. For example, by varying the stringency, i.e. varying the wash volumes, one can to select based upon for example (1) affinity, (2) on rate or off rate; (3) the wash conditions (pH, ionic strength, temperature).

The methods allow the practitioner to select ligands to the target of interest using virtually any affinity selection method. The most effective combination of analysis dimensions can easily be determined by one skilled in the art based upon the particular results desired. The claimed system is advantageous in that the direct coupling of various unit operations with μL volume connections diminishes the dilution, loss and contamination of samples by circumventing fraction collection

and manual sample transfers. A second advantage is the use of direct transfer with analyte enrichment at the inlet of down-stream columns either directly or through the use of mobile phase exchange systems. For example, an accumulator, such as a reversed phase column may be positioned between any dimensions of the system. This eliminates the manual operations of concentrating and exchanging mobile phases between separations steps.

Additionally, the methods described above can be configured so as to characterize the interaction between different ligands. These experiments can be conveniently performed by plumbing various columns in tandem mode, and without a need for attaching a label to the ligand. One or more of factors can be combined to achieve the desired result.

Thus, in certain embodiments, the practitioner can use the methods of the invention to screen ligands for their ability to bind to a certain first target molecule, and their inability to bind to a second target. This technique should be generally applicable for selecting ligands that differentiate between two different targets. The claimed methods remarkably allow the artisan to rapidly select ligands based upon their ability to bind to certain targets, and not others. This ability is especially critical in the pharmaceutical field, where potential new drugs may interfere not only with foreign or pathogenic targets, but also with host molecules. This is due to the highly conservative nature of the pathogenic and host targets. Using the claimed methods, a ligand can be selected that binds, for example, to a pathogenic target, i.e. a bacterial protein, but not to the homologous host target. This technique, referred to as subtractive screening, can be used to differentiate between any two binding characteristics. For example, the technique not only can differentiate between pathogens and hosts, but can also be used to differentiate between, a chiral or non-chiral form of a molecule; wild type target versus mutant proteins and various subclasses or variants of a target.

Alternatively, one can immobilize one subclass on a column, and introduce both the sample solution and the second subclass target molecule. The ligands which preferentially bind to the second subclass target spend more time in the mobile phase, and thus are eluted first from the column. Ligands which preferentially bind to the first subclass target will be the last to elute from

the column. Obviously, the middle eluate is a combination of varying affinities for both subclass one and two.

In other embodiments, the methods and systems of the invention encompass other techniques for selecting ligands to a target of interest. The inventors have developed novel methods not only for selecting particular ligands based on affinity or subtractive screening, but also for selecting ligands which bind to a target of interest at a particular binding site. In the latter embodiments, the invention contemplates the use of a first separation system, followed by an accumulator such as a reversed phase column. As discussed above, the methods involve two dimensions, plumbed in tandem mode.

The first dimension, a size exclusion chromatography system, is very similar to a dialysis system in which the inability of a target or receptor to penetrate a pore matrix excludes it from certain liquid elements of the system. In fact, this first dimension can be a dialysis system or any other system capable of separating target/ligand complexes and unbound components. Thus, one may contact a sample solution with the target of interest, ligands to the target will bind thereto. The solution may then contain a mixture of unbound sample components and ligand/target complex which may be introduced into an SEC column. Alternatively, one may introduce the target and sample directly into the SEC column. When one introduces this mixture to an SEC system, unbound components will diffuse into the pores of the SEC matrix, however the complex, and the target are excluded because of their size. Because the macromolecular target/ligand complex moves through an SEC column faster than the lower molecular weight components, the complex will elute from the column first. The eluate containing target/ligand complexes can then be introduced to a second dimension such as an affinity column. One immobilizes a known ligand to the binding site of interest on the affinity column. Ligands which elute off this second column will be those ligands which bind at the site of interest which were displaced by the known ligand immobilized to the column. When the eluate passes through the immobilized column, weakly bound complexes may dissociate when the separation of target and ligand occurs very rapidly, maintaining the equilibrium will become dependent on the off-rate (k_2). This means that a low

mobile phase velocity selection will depend on the equilibrium constant while at high mobile phase velocity the selection will be based on the rate constant k_2.

Thus, one may select for a ligand which will interfere with the binding activity of the natural ligand/target pair. Ligands identified by this technique are particularly well suited for use as pharmaceuticals or diagnostic aids. For example, scientists have recognized the relationship between CD4 and gp120, in those infected with the human immunodeficiency virus (HIV). Thus, it would be beneficial to obtain a ligand which can disrupt the interaction between these two molecules, thereby inactivating the active complex. Using an affinity column, one can select ligands which bind to the target of interest. Those ligands which do bind are eluted from the affinity column and introduced into another affinity column. The second column may be a size exclusion column. The eluted target/ligand complexes from the first column are then introduced to the second column, along with a known ligand. The known ligand can be any ligand known to bind at the particular epitope one is seeking a binder to. Thus, the known ligand will compete with the ligand on the target/ligand complex, and displace ligands which bind at the selected site on the target molecule.

One can envision applications of this technique wherein a DNA transcription factor is immobilized on the second column thereby facilitating the detection of a ligand which binds at the same site on the transcription factor as DNA.

Alternative techniques of obtaining the same result exist using the claimed methods. For example, to select site specific binders, one can compare the eluate from a column containing both the target molecule and a known ligand with eluate from a column containing only target molecule.

Screening based on the rate of dissociation of the ligand/target complex may be achieved in several ways. In the systems described above, the concentration of the target increases as the ligand elutes from a section of the column. Because the rate of complex formation is much higher than the rate of dissociation, it is impossible to carry out the off-rate selection in porous chromatography sorbents. The embodiment described below allows off-rate selection by

exploiting the fact that, subsequent to complex dissociation, there is a low probability that the ligand will contact a target bearing surface again before elution from the system.

In various embodiments, additional dimensions relate to selecting and recovering ligands with a preselected affinity for a target of interest. Preselected affinity refers to the ability of the ligand to specifically bind to the target molecule, i.e. the strength of the interaction between target and ligand. Typical values for the preselected affinity are in the order of 10^{-3} l/mol to about 10^{-4}l/mol at a minimum, and are preferably about 10^{-8} to about 10^{-10} l/mole. The preselected affinity value is dependent on the environment in which the ligand and target molecule are found, as well as their concentration. In some applications, a lower affinity is acceptable, while in other applications, the affinity value may be much higher. One skilled in the art can routinely determine the desired affinity constant depending on the particular target and application of interest.

It is also possible to select specific binding conditions through the selection of the mobile phase with which the column is washed subsequent to peptide binding. To determine T for a particular application, one skilled in the art can calibrate an affinity column using any of the methods known in the art. For example, one may calibrate the column by introducing a pure sample of a ligand having a known affinity constant for binding to the immobilized target. The ligand is then loaded onto the column. Serial column volumes are then passed through the column, and directly introduced into the accumulator, until one obtains the ligand with the known affinity constant K'. T can then be calculated based upon the following equation:

$$T = \frac{K'}{K_{(known)}}$$

Thus, K', also known as the retention factor is defined by the equation

$$K' = K \times (T)$$

In practice, therefore, if one desired to obtain a ligand having an affinity constant K of 10^{10}, T could be set at, for example, 10^{-8}M. The ligand having the preselected affinity K will be obtained upon passing approximately 100 column volumes of solvent through the column.

$$10^{10} = \frac{K'}{10^{-8}}$$

After the sample is passed through the column so that ligands bind to the target molecules, a series of column volumes of solvent are passed through the column, wherein n is a number of column volumes between 1 and 10,000. A subset kp of the volumes exiting the column are then passed to a ligand accumulator to immobile thereon ligands having the preselected affinity K.

Elution of the affinity bound material is typically performed under conditions of high salt or acidic pH. Once eluted, the soluble sample can be analyzed to determine the presence or absence of the ligand to the target of interest, or to characterize a ligand to the target of interest. Analysis may be performed by any method suitable for the determination of components.

In yet other embodiments, the invention relates to a method, system and apparatus for separating ligands in a sample into groups having similar affinity constants. One skilled in the art can determine for each application the range of affinity constants to be included in each group depending upon the desired result.

In this embodiment, the artisan first selects which groups of affinity constants are desired. For example, if T is 10^{-5}, and volumes 20-30 and volumes 500-800 have been collected from the accumulator, then the K for the ligands in each group can be determined as follows:

the presence $$K = \frac{2 \times 10^1}{10^{-5}} = 2 \times 10^6 \text{ and}$$

$$K = \frac{3 \times 10^1}{10^{-5}} = 3 \times 10^6$$

and the K for the 500 -800 volumes will be between

$$K = \frac{5 \times 10^2}{10^{-5}} = 5 \times 10^7 \text{ and}$$

$$K = \frac{8 \times 10^2}{10^{-5}} = 8 \times 10^7$$

Thus, in a basic embodiment, the methods, apparatus and system of the invention allow one to detect the presence of, or determine the affinity constant of biomolecules. Varying concentrations of ligands in a sample can be introduced to a column having a fixed concentration

of target. The concentration of the unbound ligands varies with the initial target concentration, and following automated data manipulation, the affinity constant can be determined.

The screening procedure can be used repeatedly to detect ligands or analytes from these sample libraries, having a preselected affinity. One may prefer the binding constant gradient obtained for one ligand to the target of interest to that of another, depending upon the desired elution conditions. For example, the strength of binding in a series of solutions containing methanol at increasing concentrations or solutions with increasing salt concentrations simulating elution gradients can be used. In this way, one can evaluate the comparative behavior of a number of ligands at a multitude of elution conditions.

The target immobilized column may be directly coupled to an accumulator, such as a reverse phase column. The method in various embodiments employs a tandem column chromatographic technique in which the target-ligand complex captured by the first column is dissociated and eluted directly onto an accumulator such as a reverse phase column for caption of the dissociated ligands. The bound complex can then be dissociated from the column, and introduced into the RP column. The accumulator should be suitable for selecting the bound ligand-target complexes eluted from the chromatography column. If the accumulator is, for example, a size exclusion chromatography column, one can assume that if the flow rate through the SEC column is rapid, there is little time for the dissociation of the target-ligand complex. At a slow flow rate, the opposite is true. As discussed in greater detail above, there is a direct correlation between the amount of washing of the complex on the first column with the relative affinities of the ligands.

The methods of the invention also encompass the preparation of pharmaceutically active compositions wherein a ligand in a sample having a preselected affinity K for a target molecule of interest is identified. The ligand is identified by loading a column with a known concentration T of target molecules; passing a sample through the column to bind ligands in the sample, and passing through the column a series of column volumes of solvent. A subset of the column volumes exiting the column are then passed to an accumulator to immobilize thereon ligands having the preselected affinity. The ligand is then eluted, and the ligand, or a derivative thereof is

used to generate a biologically active component for incorporation into a pharmaceutical preparation. If desired, one may incorporate appropriate adjuvants therapeutic carrier, etc. A pharmaceutical preparation obtained by any of the above methods can provide a method of treatment by administration of the preparation.

The methods of the invention include methods for detecting a ligand having a desired high affinity K for a preselected target molecule when the ligand and the target molecule are present together in preselected solvent conditions. The ligand may be present in a heterogeneous sample comprising a multiplicity of ligand species. These methods comprise immobilizing a target molecule onto a column, passing the sample through the column under conditions to promote binding of ligands in the sample to the target molecules, and then passing through the column a series of column volumes of solvent defining the solvent conditions. One then passes a subset kp of the column volumes exiting the column through a ligand accumulator to immobilize ligands having the desired high affinity. The ligands are then eluted, and optionally, identified. The ligand obtained may be characterized by a high affinity K for the target molecule equal approximately to kp/T where T is the concentration of target molecules in the column. Optionally, the sample may be passed through the column to promote the binding of ligands under solvent conditions different from the preselected solvent conditions.

In other aspects of the invention, a ligand having a high on-rate, Ko, when the target molecule and the ligand are present together in preselected solvent conditions is detected. The ligand may be present in a heterogeneous sample comprising multiple ligand species, at least one of which binds a preselected target molecule with an affinity of at least about $10^4 M^{-1}$. As in other embodiments, the target molecule is immobilized on a column. A heterogeneous sample in a solvent defining preselected solvent conditions is provided, and then passed through the column at a high linear fluid velocity so as to minimize the residence time of ligands in the sample. Thus, one selectively binds high on-rate ligands to the target molecules, in preference to other ligands in the sample. One then elutes the column to produce an output, and obtains or identifies the high on-rate ligand. One may optionally pass the output through a ligand accumulator, and then elute the accumulator to produce an output rich in the high on-rate ligand.

C. THE INTERFACES

The system and apparatus of the invention may also include a coupling interface for capturing the eluent from one dimension and introducing it to one or more different dimensions. The interface may optionally contain a buffer system to effectively desalt, dilute or remove organic solvent from the eluent of one dimension prior to loading in the next dimension. Thus, in some situations, one may incorporate a buffer exchange in the interface. The buffer exchange may be a mixed bed matrix, packed with cation and anion exchange sorbent. Alternatively, the buffer exchange may comprise a separate column for each of the cation and anion exchange sorbent.

Tandem columns of cation exchanger and anion exchanger, or a mixed bed exchanger can be used to capture biomolecules from the eluent of a column. Thus, for example, if one eluted a desired ligand from any column in the system with an acid, the eluant could be directed into a tandem buffer exchange to alter the pH prior to introduction to the next column. The eluant may first be introduced into a cation exchange column which will capture the ligands from the eluant. The cation column is then washed with a neutral pH buffer, and the desired ligands captured onto the subsequent column, i.e. an anion exchange column. The ligands can then be eluted off this second column with a buffer or solvent optimized for introduction into the next dimension of the system.

The buffer exchange interface is particularly valuable in multi-dimensional systems where the elution buffer of, for example, an affinity column, is not suitable for introduction into a mass spectrometer. Thus, the desired ligands are washed from the affinity column, passed through a cation column and an anion column prior to introduction into the mass spectrometer.

In an alternative embodiment, the claimed invention relates to the tandem use of an affinity column, and a column having immobilized thereon an enzyme for digestion. Thus, a desired ligand may be captured in the first column, an affinity column, and eluted with acid. The ligand in the eluant may then be captured on a cation exchange column. The desired ligand can then be eluted off of the cation exchange column with a buffer optimized for the next column, i.e. a trypsin column.

Upon passing through the trypsin column, the ligand is digested and may, for example, be captured on a reversed phase column.

One can manipulate either the pH or salt to elute different columns; various configurations of the anion and cation columns are contemplated and can be configured so as to optimize the buffers for subsequent dimensions. It would be possible that this technology could be extended to quality control and process monitoring in biotech applications, the study of therapeutic protein and drug metabolism, and large scale screening programs of environmental and clinical importance.

Preferably the interface further comprises a multi-valving system capable of the direct transfer of solutes from one dimension to the next without dilution or manual intervention. The claimed invention may have both the buffer interface described above, and the valving interface, depending on the desired application.

The claimed invention encompasses a valving system which will avoid the laborious process of collecting solutes from each column and reintroducing them onto a second column. In several embodiments, the valving system is a "sample splitter" which allows a liquid chromatography eluent to be interfaced to a mass spectrometer (Figures 1 and 2). The advantage of the claimed interface is that the flow rate of the mass spec (MS) and the liquid chromatography column (LC) can be independent and variable, i.e., the MS and LC rates can be independently optimized while the MS sampling is decoupled from the LC flow rate. The invention also contemplates an interface capable of simultaneously sampling LC eluant from multiple LC columns and desalting an LC eluant sample prior to introducing the sample to the MS.

For example, the interface of the invention may be an interface for sampling the eluant of a LC stream and injecting into another LC stream, or a detector such as a mass spectrometer. Thus, Figures 1 and 2 depict an embodiment of an analytical chemistry system 10, featuring a liquid chromatography/mass spectrometry sample splitter interface. The liquid chromatography column 12, having a liquid stream 14 driven at the desired rate for the liquid chromatography column by a pump 16, and a mass spectrometer 44 having a liquid analysis stream 38 driven at the desired rate for the mass spectrometer by a precision pump 36. A

sampling valve 22 is cycled at a predetermined sampling rate to insert a sampling volume into the liquid chromatography stream to take a sample of the liquid chromatography eluant, and then insert it into the mass spectrometer stream to analyze the sample.

More specifically, the liquid chromatography eluant 18 from the LC column 12 flows into an input port 20 of a sampling valve 22 and out through an output port determined by the selected position of the valve. An LC detector 26 can be connected to an output port 28 of the sampling valve 22 to accept the LC eluant from the LC column directed through the sample valve. Alternatively, the LC detector can be placed between the LC column and the input port 20 of sampling valve 22 with similar results.

Sampling valve 22 can, for example, be a rotary multi-port valve capable of two possible by-pass configurations and controlled by a sample controller 24. Figure 1 shows the sampling valve in a first position A, and Figure 2 shows the sampling valve in a second position B. In valve position A the LC stream sampling position, the eluant 18 from LC column 12 enters the sampling valve through input port 20 and exits the valve through output port 30. Output port 30 is connected to another input port 34 by a length of tubing 32 defining a sample loop having a predetermined sample volume. In position A, LC eluant flow through the sample volume into input port 34 and is directed to output port 28 for detection.

A MS precision pump 36 pumps MS analysis stream 38 into another sampling valve input port 40, and MS 44 accepts its analysis stream from another sampling valve output port 42. In valve position A, the MS stream 38 goes through input port 40 directly to output port 42 and to MS 44.

Upon switching the sampling valve 22 from position A to position B (Fig. 2), the MS infection position, the predetermined sample volume of LC eluant is trapped in the tubing 32, and transferred into the MS analysis stream 38. Specifically, in position B, sample valve 22 directs the LC eluant through input port 20 directly to output port 28 and to the LC detector 26. However, the MS analysis stream 38 is now directed through sample loop tubing 32 into MS 44, thereby injecting the trapped sample volume of LC eluant in tubing 32 into the MS analysis stream.

In operation, the flow rate of the stream for the LC column 12 can be substantially different than the flow rate of the analysis stream for the MS 44. For example, a typical LC flow rate can be greater than 100 µl/ min. An MS flow rate can typically range form 1 to 10 µl/min. Furthermore, either the LC or the MS, or both flow rates can be dynamically variable.

Sample controller 24 operates to cycle the sample valve 22 to split the LC flow such that only a small portion of the flow from the LC eluant stream is injected into the MS analysis stream. The sample volume of the sample loop tubing 32 can be selected to have a negligible effect on the LC stream, yet be sufficient for the MS analysis. For example, a 1 µl sample volume removed from the LC stream would have a negligible effect on a 100 µl/min. LC stream, yet would be a sufficient sample for a 10 µl/min. MS stream.

When sampling valve 22 is cycled to position A, the LC eluant from the LC column 12 is caused to flow through sample loop tubing 32, filling the sample volume. Upon cycling sampling valve 22 from position A to position B, the sample volume of LC eluant trapped in the sample loop tubing 32 is pushed out of the loop to the MS at the MS flow rate of, for example, 1 to 10 µl/min. At an assumed MS analysis stream flow rate of 10 µl/min., 166 nl of the MS analysis stream are driven through the sample loop 32 sample volume every second. When the sampling valve is returned to position A, any residual sample left in the sample loop 32 is driven into the chromatography detector 26, fraction collector or waste, depending on the configuration of the system.

In operation, sampling valve 22 is cycled repeatedly to capture and transfer an aliquot of LC eluant to the MS analysis stream several times over each LC peak. By repeatedly sampling throughout the chromatographic peak of the sample, it is possible to reconstruct the chromatography from these discrete samples.

Depending on the desired application, the sample valving system may be configured to simultaneously monitor two or more liquid chromatography streams.

The multi-dimensional apparatus of the invention may also consist of an interface such as a fluid handling system with pumps for delivering the samples to the various dimensions, one or more chromatography columns and a mass spectrometer for detection. The system and apparatus

of the invention may have a software interface, i.e. controller, which allows for a wide variety of assay formats, and may optionally include a spectrophotometric detector. The software interface may be tailored to a broad range of specifications and comprises three functional areas: instrumental control, methods development, and analysis. The instrument control may provide a graphical interface to each physical element of the system, from buffer selection to sample preparation, through to detection and fraction collection. The status of the system may be continuously monitored and displayed on the computer screen.

D. DETECTION

Any method of detection known in the art is suitable for use in the claimed invention. Thus, ligands, or target molecules may be labelled to render them detectable. Thus, either the target or the ligand may be labelled with a detectable moiety such as enzymes, fluorophores, chromophores, radioisotopes, electrochemical moieties and chemoluminescent moieties. Additionally, the invention contemplates a composition comprising a first binding partner having a detectable moiety which is intrinsic, e.g. a functional group capable of detection.

Additional methods of detection include, for example, any apparatus for obtaining mass-to-charge ratio, including, but not limited to: matrix-assisted laser desorption ionization/plasma desorption ionization, electrospray ionization, thermospray ionization, and fast atom bombardment ionization. Additionally, any mode of mass analysis is suitable for use with the instant invention, including but not limited to: time of flight, quadrapole, ion trap, and sector analysis. The preferred method of detection and analysis is an improved time of flight instrument which allows independent control of potential on sample and extraction elements, as described in copending USSN 08/446,544 (Atty. Docket No. SYP-111, filed May 19, 1995).

The methods development component allows the user to create automated assay methods, including setup of injection sequences and sample preparation. Dilution and derivatization may be included in sample preparation.

Assay analysis allows the quantification of individual runs, and may incorporate parameters such as standard curves or dose levels to be prepared. Thus, a standard curve may be developed from a set of assays by automatically measuring the detector response and fitting this

result to the injected sample quantity. This means that an entire set of assays, including standard curve generation, can be conducted with minimum operator intervention thus increasing the accuracy and decreasing the contamination of the assay.

It will be understood by those skilled in the art that the claimed invention contemplates not only detecting the presence of a ligand having a preselected affinity, but also recovery of that ligand, and its subsequent use. For example, ligands having a preselected specific affinity for a target toxin may be used as scavengers, in vivo and in vitro. The ligands thus obtained can be used therapeutically. Additionally, the methods, apparatus and system of the invention can be used to find lead compounds for drug discovery. The claimed invention relates not only to lead compounds, but also to the use of those lead compounds to identify new drugs. The invention also contemplates preparations prepared using the ligands of the invention.

The kits of the invention include apparatus capable of performing at least one "dimension" or method described above. It is preferable that the kits be capable, for example, of the automated detection of a ligand in a sample, having a preselected affinity K for a preselected target molecule. Such kits may contain a column having a known concentration of the preselected target molecule immobilized thereon, and an accumulator capable of receiving eluate from the column, and immobilizing thereon a ligand with the preselected affinity K. The kits may be adapted to any or all of the methods described above, and claimed herein.

The kits may optionally be configured to detect ligands as well as to analyze, and obtain said ligands, and may include, for example, an interface for rendering a sample solution compatible with additional analyses. The kits of the invention may be used to identify and obtain ligands in samples. The ligands, or derivatives or modifications thereof can be used for a variety of purposes, such as lead compounds for drug discovery. Optionally, one may use the ligands or modifications or derivatives thereof to prepare a pharmaceutically active composition.

Pharmaceutically active compositions of the invention are prepared by identifying ligands in samples having a preselected affinity K for a target molecule of interest. The ligand may be identified by loading a column with a known concentration T of target molecules, passing a sample through the column to bind ligands in the sample thereto, and then passing a series of column volumes of solvent through the column. A subset of the column volumes exiting the column can be introduced to an accumulator, to immobilize thereon ligands having the preselected affinity, and eluting the ligand.

Compositions of the invention may optionally include adjuvants.

Advantages of the present invention include speed, reproducibility and automation. It is preferable to use particles capable of perfusion chromatography since perfusion particles allow the system to operate at very high flow rates while maintaining both high sample loading capacity and chromatographic resolution.

As will be understood from the above description and the examples given below, the claimed invention is amenable to numerous configurations which may be chosen on the basis of the desired application or the sample. To further exemplify the invention, several configurations are enumerated in more detail below. In certain embodiments, as depicted in Figure 3, the apparatus of the invention 110 comprises a sample input 112 for introducing sample into the apparatus 110 into a first column 114 to partition, based on a first physico-chemical property, candidate ligands, or complexes thereof with a target molecule, to generate a first exit stream 116. First exit stream 116 is optionally directed to a second column 118 to partition candidate ligands based on a second, different physico-chemical property, to generate a second exit stream 120.

In various embodiments, there is optionally an interface 122 between the first column 114 and the second column 118 to condition the solvent in the first exit stream 116 for introduction into second column 118. Optionally, first multi-valved splitter 124 may be positioned between the first column 114 and second column 118 to direct the first exit stream 116 to the interface 122, to the detector 125, where, if ligand is present, exit stream 116 is reintroduced to valve 124, or if no ligand is present, exit stream 116 is directed to waste stream 126.

The multi-valved splitter 124 can be a rotary multi-port valve capable of two or more possible by-pass configurations and controlled by a sample controller 128.

Second exit stream 120 is optionally introduced into a second multi-valved splitter 130 which directs the second exit stream 120, into a third column 132. A sample of the third exit stream 134 containing a selected ligand may optionally be inserted into a third sample splitter 140, and then into a mass spectrometer 136 for determination of the charge to mass ratio of the ligand. A display 138 may be connected thereto.

In different embodiments, first column 114 is an affinity column for partitioning a library based upon a first physico-chemical property, and second column 118 is another affinity column to partition the first exit stream 116 based upon a second physico-chemical property. Third column 132 may be a reversed phase column capable of accumulating the desired ligand thereon prior to eluting the desired ligand into third exit stream 134.

In an alternative embodiment, first column 114 can be a dialysis or size exclusion system capable of partitioning based upon size. Second column 118 is an affinity column capable of partitioning based upon a second physico-chemical property.

In another exemplary configuration of the claimed apparatus, (Fig. 4) various solvent reservoirs 146 are connected via a valve 148 to a valve 150. Sample is introduced through the sample input 112, and combined with the solvent at valve 150. The solution is then introduced into first column 114, and the exit stream 116 can then be directed through a valve 124 to a detector 125, where, if ligand is present, exit stream 116 is reintroduced to valve 124, or if no ligand is present, exit stream 116 is directed to waste stream 126. Alternatively, or additionally, exit stream 116 may be introduced to a second column 118 suitable for diluting, desalting, or

removing organic solvent from the exit stream 116 prior to reintroduction to the system through valve 124. Exit stream 152 is then introduced into third column 132. Third exit stream 134 can then be introduced to a splitter 140, and a sample directed to the mass spectrometer 136, and ultimately, the information is transmitted to an output display 138.

EXAMPLES

Example 1: Screening of a Synthetic Peptide Combinatorial Library (SPCL) using an antibody against β-endorphin as a target

Example 1A: Preparation of the Target Immobilized Affinity Column and the Control Column

Monoclonal antibody (mAb) chosen (mouse IgG2a, clone 3E-7, Boehringer Mannheim, Indianapolis, IN) was raised against human β-endorphin and recognizes the amino terminus of β-endorphin, YGGFL. The purchased mAb (280mg resuspended in 1ml H_2O) was passed over an XL cartridge (2.1 x 30mm) consisting of protein-G coupled to POROS® perfusion chromatographic media (PerSeptive Biosystems, Framingham, MA) by making 10 x 100ml injections on a BioCAD™ 20 Workstation (PerSeptive Biosystems, Framingham, MA). Protein-G binds to the Fc region of antibodies with high affinity. The mAb was subsequently cross-linked to the protein G using the standard methods and materials provided with the XL column. In brief, this consisted of passing 14 ml of cross-linking solution (100 mM triethanolamine, pH 8.5, 7.8 mg/ml dimethyl pimelimidate (DMP)) over the column at a flow rate of 0.5 ml/min. The cross-linking reagent was quenched by subsequent injection of 2 ml of 100 mM monoethanolamine, pH 9.0. The sensor cartridge was washed with PBS, pH 7.4 at 0.5 ml/min for 2 min followed by a further injection of 2 ml of quench solution and washed as above. The antibody was efficiently immobilized to the column as demonstrated by the lack of reactivity to coomassie stain upon SDS-PAGE of the flow through. A second XL column (without antibody) was treated with cross-linking reagents and washed as for the affinity column for use as a "control column".

Example 1B: Control Screening of the Immobilized Target with the Known Epitope

The target immobilized affinity column prepared above was plumbed in tandem mode to a vydac reversed phase C-18 column on a BioCAD™ Workstation using the configuration shown in Figure 5. This configuration allowed independent equilibration and washing of the affinity and

vydac columns while allowing in line elution of material bound to the mAb-column directly onto the vydac column. The reverse phase vydac column could then be eluted independently of the affinity column.

To investigate the binding of the immobilized antibody to its epitope YGGFL, the synthetic peptide YGGFL (Sigma Chemical Co., St. Louis, MO; 1 mg/ml in PBS, pH 7.4) was injected onto the affinity column (flow rate 0.2 ml/min). After washings with 10 column volumes (CV) of PBS, pH 7.4, the bound peptide was eluted from the affinity column with 12 mM HCl, directly onto the vydac C-18 column. This reversed phase column was then developed with a gradient of acetonitrile (4% ACN/12 mM HCl to 80% ACN/6 mM HCl over 18 min; flow rate 1 ml/min). Both columns were re-equilibrated in their respective starting buffers prior to the next injection.

Example 1C: Synthesis of the Soluble Peptide Combinational Library (SPCL)

The SPCL of general formula NH_2-XXXFL-COOH (wherein X represents any of the natural L-amino acids except cysteine and tryptophan) was synthesized as described (See Sebestyen, F. et. al. Bioorganic Med. Chem. Letts. 3; 413-418 ,1993) According to standard practice, the peptides were cleaved from the resin using "reagent B" and precipitated into ether. Calculations of the amount of each peptide gave a theoretical quantity of 70 nmols of each of the approximately 5832 potential sequence possibilities within the library (assuming equimolar coupling of each amino acid at each step in the synthesis and relatively equal recovery of each peptide for the ether precipitation). Sequences of either individual peptides obtained by immunoaffinity purification or "pool" sequence of the crude library were obtained by sequencing on a Hewlett Packard G1000A protein sequencer using standard HP2.2 chemistry (Hewlett Packard, Palo Alto, CA).

Example 1D: Control Binding Experiment

As a first step, whether the mAb immobilized onto the XL cartridge retained its ability to bind the peptide YGGFL was investigated. The peptide solution (20 nmol) was injected onto the affinity column and then unbound peptide was removed by washing the column with PBS, pH

7.4. Peptides affinity captured by the antibody was eluted from the "target column" directly onto the C-18 column for resolution. The amount of the peptide recovered, as calculated from the peak area, was approximately 1.2 nmoles. The theoretical capacity based on the quantity of mAb loaded onto the affinity column was 3.8 nmol demonstrating that approximately 25% of the binding sites of the mAb are available in a conformationally active form.

Example 1E: Determining the Capacity of the Target Immobilized Affinity Column

The capacity of the column was examined by injecting increasing larger quantities of the peptide (YGGFL) utilizing the loading template of the BioCAD™ Workstation. The amount of the bound peptide (as calculated from the peak height) reached saturation at about 1.2 nmoles. Interestingly, the amount of peptide bound to the antibody was independent of the flow used to inject the peptide. Increasing the flow rate from 0.2ml/min to 5 ml/min did not affect the recovery. This result suggests a rapid interaction of the peptide with the antibody during the loading process when a perfusive packing material and elution conditions are used. The EC_{50} value (50% of the saturating amount) for the peptide is approximately 30 nmols. This value correlates well with the affinity constants determined previously for binding of YGGFL peptide to 3E-7 by competitive radiolabelled binding assays (See Lam *et. al.*, *Biorganic Med. Chem. Letts. 3:*419-424 (1993)).

Example 1F: Screening of the SPCL using the Target Immobilized Affinity Column

The diversity of the library was first assessed by running a small aliquot on the vydac C-18 column under the same conditions used for elution of bound material from the XL column. The large number of peaks exhibiting significant absorbance at the indicated wavelength are suggestive of the diversity of the library.

Using the conditions established for purified YGGFL binding to the mAb column, the XXXFL library was screened for moieties recognized by the target immobilized mAb affinity column. The library (containing 2.8nmols of each peptide) was loaded onto the "target column" , the unbound material was washed with 10 Column volumes of PBS, pH 7.4. Finally, the affinity bound material was eluted directly onto the vydac C-18 column with 12 mM HCl. Elution of the C-18 column (with a 4-80% ACN gradient as described above) revealed approximately 10-12 resolvable peaks. The elution profile shows that, one of the peaks observed exhibits a retention

time comparable to that observed for pure YGGFL. Using the chosen wash conditions (10 CV) for the immunoaffinity column, we were able to selectively discriminate a single moiety from a library with potentially greater than 5800 individual peptides. The identity of isolated YGGFL was confirmed by mass spectrometry and peptide sequencing.

Example 1G: Control

In order to confirm the specificity of the target immobilized affinity column for YGGFL, samples of the purchased peptide and the library (XXXFL) were analyzed in a parallel experiment using the control column prepared in Experiment 1. The chromatogram was identical except for the peak corresponding to YGGFL.

Example 2:

Screening of a peptide combinatorial library employing Endotoxin (lipopolysachharide) as a target

Example 2A: Preliminary Experiments

Several agents, including some peptides, bind to endotoxin and reduce the lethal effects of this agent in animals. However, these agents are of limited use because of their inherent toxicity. A linear SPCL of the general structure XXXFL (similar to the core region of Polymixin B (PmxB) was screened for moieties capable of binding to LPS and a cyclic library (of the general formula CXXXC) which is cyclized by virtue of the disulphide linkage between the two cysteine molecules.

We used a tandem column method employing POROS® columns on a BioCAD™ 20 Workstation. Column 1 was a weak anion exchange column (PI/M; 4.6 x 100 mm) while column 2 was a reverse phase column (R2/H; 4.6 x 100 mm). A weak anion exchange column was chosen for column #1 since it has previously been demonstrated that agents capable of binding to LPS possess a degree of cationic character and hence would not necessarily be expected to bind to this column. It was initially demonstrated that the molecules pentamidine and polymyxin B (previously demonstrated to bind to the lipid A region of LPS) were retained on column 1 under the conditions used only after preincubation in the presence of LPS. LPS, however, would be

expected to interact with this column due to the presence of two charged phosphate groups at the lipid A region of the molecule. Hence cationic molecules capable of binding to LPS would only be retained on column 1 if they were bound to this molecule. Material retained on column 1 was eluted directly onto column 2 where it was captured. Elution of column 2 allowed the resolution of peaks arising from column 1.

Example 2B: Synthesis of the XXXFL Library

The library XXXFL was made on Fmoc-Leu WANG resin using standard procedures in an Advanced Chemtech librarian peptide synthesizer. Prior to screening the library (consisting of approximately 5800 pentamers) it was divided into an anionic and cationic fraction based on retention on the weak anion exchange column (material binding to the column when injected in 50 mM tris, pH 6.7 was designated as the anionic fraction, while that passing through was the cationic fraction). Only the cationic fraction was screened using this paradigm. However, the cationic fraction represented > 2/3 of the total library based on peak area for the two fractions.

Example 2C: Screening of the XXXFL Library

Screening the library for binding to LPS was performed as follows. The cationic fraction of the XXXFL library was incubated (30 min/RT) with 1-3 mg/ml of LPS (serotype O55;B5; Sigma Chemical Co., St. Louis, MO) in 50 mM Tris, pH 6.7. At this time the incubation mixture was injected and run over column 1 on the BioCAD™ Workstation (equilibrated in the same buffer). The column was run at 4 ml/min. After washing with the appropriate number of column volumes of equilibration buffer (1 CV = 1.66 ml), the column was purged and eluted (using 8 mM HCl, 1M NaCl) directly onto column 2 by switching the latter column in line with column 1 during the elution process. Material captured on column 2 was eluted using a gradient from 12 mM HCl in water to 80% ACN, 6 mM HCl. Peaks were collected, further purified by rerunning on a vydac C-18 column (4.6 x 250 mm), and analyzed by Mass Spectrometry (on a Voyager™ MALDI-TOF instrument; PerSeptive Biosystems, Framingham, MA) and peptide sequencing (Hewlett Packard). Peptides identified were synthesized and binding of these molecules to the

Lipid A region of the LPS was confirmed by measuring the ability of PmxB or Pentamidine to compete with the peptide for this site.

Furthermore, increasing the number of column volumes used to wash column 1 prior to elution onto column 2 resulted in a decrease in the subsequent recovery of these molecules, presumably due to dissociation of these agents from the LPS while on column 1. The number of wash volumes required to reduce the peak for these agents correlated with the reported affinities of these agents for LPS; PmxB (Kd 0.4 mM) was reduced 50% after 42 CVs washing of column 1 while pentamidine (Kd 100 nM) required a higher number of washes for a similar reduction (46 CVs). Using this methodology we could rapidly examine which peaks from the library were capable of binding to LPS with the highest affinity since, upon exposure to higher numbers of wash volumes only the peptides with higher affinity for the target were retained. The relative reduction in the peak area (or height) for each peak eluted from the reverse phase column when plotted against the number of CVs used to wash column 1 could be used to distinguish the peaks with higher affinity since these showed a lower rate of reduction under the same conditions. Three such peaks from the XXXFL library and 3 peaks from the CXXXC library were further purified and characterized by MS and peptide sequencing. Results from the sequencing data for the XXXFL candidates suggested the structures RRRFL, RRKFL or KKRFL. The latter peptide has been synthesized and demonstrated to bind to LPS using the same paradigm above. However, while this peptide is displaced by Pentamidine in a competition experiment, its affinity for LPS is much lower than the latter molecule. The other peptides will be synthesized and studied in a similar manner although another approach being considered is that a sublibrary of the format ZZZFL (where Z represents either R or K) be screened to identify the member with the highest affinity. Candidates purified from the cyclic library are still awaiting sequencing.

Example 2D. Affinity-Based Screening - Selection of High Affinity Binders - Effect of Wash Volumes

Tandem columns consisting of an affinity column plumbed in-line to a reversed-phase (RP) column can be used to screen libraries to select for binders of a known affinity based on the volume of washing of the affinity column.

A BioCAD™ Workstation was plumbed in the tandem column configuration. Column one was an immobilized rHsp70 affinity column (2.1 X 30 mm) and column two was a POROS® R2 reversed-phase column (2.1 X 100 mm). The affinity column (column one) was washed with screening buffer before the library (natural protein digest library [PDL], 100 µg) was injected onto the affinity column at a flow rate of 0.2 ml/min. The affinity column was then washed with screening buffer in increasing numbers (5, 10, 20, & 40) of column volumes (CVs) at 0.2 ml/min. (1CV = 100 µL) The POROS® R2 column was then switched in-line downstream of the affinity column. Bound material was eluted off the affinity column with acid directly onto the in-line RP column. The affinity column was then taken off-line and washed back into screening buffer. Finally, the POROS® R2 column was eluted with an ascending acetonitrile gradient (0-80%) in TFA (0.1%).

Figure 6 shows the RP column portion of the experiment, i.e., elution onto an in-line RP column after selection by a rHsp70 affinity column from PDL after different number of wash volumes. At 40 CVs only a single peak is selected ("**" representing a high affinity binder) suggesting that the protein represented by that peak has a high affinity for rHsp70. At lower wash volumes, e.g. 5 CVs, other peaks ("*" representing a low affinity binder) are seen. These peaks are absent at 10 and higher CV washes, suggesting that they are low affinity binders.

Example 2E. Subtractive Screening-Selection of Target Specific Binders

A multi-target column format can be used to screen ligands for their ability to bind to a certain target and for their inability to bind to a second target in a single process. This technique should be generally applicable for selecting binders that differentiate between two different targets. For example binders can be selected that differentiate between wild type and mutant protein. For another example, ligands can be selected that bind to a pathogenic target but not to the homologous host target. Subtraction can be done in one chromatographic process or in parallel chromatographic runs with subtraction done at the analysis level.

A library has been screened using a SEC subtractive screening protocol with human Hsp70 as the host protein and its E. coli counterpart, DnaK as the pathogen target. A BioCAD™ Workstation was plumbed in the tandem column configuration. Column one was a size exclusion column (SEC) and column two a POROS® R2 reversed-phase (RP) column (2.1 X 100mm). Protein (50µg) was pre-incubated with library (100µg) and samples (100µL) were injected onto the SEC at a flow rate of 1 ml/min. The protein peak was either collected for mass spec. analysis

or the POROS® R2 column was switched in-line downstream of the SEC. The protein peak was cut directly onto the in-line RP column, and the SEC was taken off-line. The POROS® R2

column was then eluted with an ascending acetonitrile gradient (0-80%) in TFA (0.1%). Finally, the SEC was equilibrated back into screening buffer.

Total peptides eluted from rHsp70 and from DnaK were analyzed by MALDI-TOF. Figures 7A, 7B, 7C and 7D show the MALDI-TOF spectra for such experiments. A number of peaks are present in the rHsp70 sample incubated with the PDL (Fig. 7A) that are not seen in the control incubations with rHsp70 (Fig. 7B) or PDL (Fig. 7C) alone. This indicates that these peptides are binding to rHsp70 itself or that there is insufficient separation of bound and unbound library. DnaK incubated with PDL (Fig. 7D) also binds peptides originating from this library. DnaK binds a different ensemble of peptides, although some are in common with rHsp70.

Example 2F. Bimolecular Screening-Selection of Site-Specific Binders

Tandem columns consisting of an affinity column plumbed in-line to a RP column can be used to screen libraries for ligands that bind to a specific site on the target molecule via comparison of the eluants from a column containing both target molecule and a known ligand with the eluants from a column containing only target molecule.

The lectin Concanavalin A (Con A) has been used. Ligands were screened for specific binding to the sugar site of Con A as an example of the bimolecular approach to screening a mixture of components for interaction with a target. Biotinylated succinyl Con A was immobilized onto a streptavidin POROS® support (BA cartridge, 2.1 X 30 mm). A library consisting of the sequence XXXXX (where X represents any of 20 natural amino acids excluding cysteine) was passed over the affinity column, and material interacting with this support was subsequently eluted and captured on a RP column. In order to identify ligands that had specificity for the sugar-binding site, the peptide library was passed over the test column in the presence and absence of a ligand for Con A (methyl-α-D-mannopyranoside, 33 mg/ml). After exposing the column to 10 CVs of CAB buffer (0.2 ml/min.), the remaining peptides were acid eluted onto a RP column. These peptides were eluted from a RP HPLC column using an acetonitrile gradient and the fractions were collected, pooled and sequenced. Recovery of each amino acid was expressed as a percentage of the total amount of amino acids recovered at each cycle of sequencing (AA%). Amino acid enrichment was then expressed for each amino acid as follows:

$$\text{AA enrichment (Cycle X)} = \frac{\text{AA\% (cycle X) from test column (in absence of sugar)}}{\text{AA\% (cycle X) from test column (in presence of sugar)}}$$

The recovery of each AA from each cycle could thus be ranked and the sequence of the 5-mer peptide with the sequences exhibiting the greatest recoveries of AA at each cycle was 'named' from this data.

Figure 8 shows the results of this data. From this data we 'named' the peptide HHRSY as being composed of the amino acids that showed the greatest increase in AA% when the column lacked sugar relative to its AA% in the presence of sugar. Synthesis of this peptide and characterization of its ability to bind revealed that it was capable of binding specifically to Con A immobilized on the column with little binding to the control column. Furthermore, this peptide could be displaced somewhat by inclusion of a high concentration of the competing sugar ligand. These data suggest that it is possible to identify ligands for a specific site on a molecule by using this bimolecular approach to screening.

Example 2G. IgG Purification

Immobilized protein A and protein G are traditionally used for purification of immunoglobulins (IgG) from serum, ascites, hybridomas and cell culture supernatents. This example provides an alternative approach to proteins A & G, since immobilized proteins tend to leach from the column and are incompatible with the acidic conditions required for elution of bound IgG.

To screen for IgG as target, peptide libraries were generated from each of the following sources: 1) Natural Peptide Libraries of generic proteins, 2) Polyclonal Antibody Libraries, and 3) Protein A and G digests. Each of the above libraries were screened for peptides that bound specifically to mouse IgG (whole molecule) or mouse IgG, Fc fragment. One of two screening procedures were followed: i) Solution phase peptide(s) screening or ii) solid phase peptide screening. Solution phase peptide screening involved incubating the peptide library with mouse IgG (whole or the Fc fragment) in solution and separating the IgG and bound peptides from the unbound peptides by and further separation of the protein and bound peptides by conventional reversed phase chromatographic techniques under acid conditions. Solid phase peptide(s) screening involved passing the peptide library through an immobilized IgG column under physiological conditions and eluting the bound peptides into a RP HPLC column under acidic

conditions. The peptide(s) selected by either one of the above screening methods were characterized by mass spectrometry (MALDI-TOF) and Edman sequencing methods. The peptide(s) were then immobilized on POROS® media and evaluated for specificity, selectivity and capacity to bind IgG from serum. Various coupling chemistries (direct synthesis or off-line immobilization), the effect of ligand density, the activation chemistry, and the nature of interaction with IgG were investigated for some of the selected peptide(s).

Example 3: Screening

All proteins and reagents for buffers were obtained from Sigma Chemical Co (St. Louis, MO) unless otherwise specified. Anti-IgG (Fc specific) antibodies were purchased from Biodesign International (Kennebunk, ME). Screening by SEC and RP Chromatography was performed on the INTEGRAL™ chromatography Workstation (PerSeptive Biosystems, Inc., Framingham, MA). Screening by immobilized target (IgG) column in tandem with RP column was performed on the BioCAD™ 20 Workstation (PerSeptive Biosystems, Inc., Framingham, MA). Size exclusion column (Superdex 200 HR 10/30, molecular exclusion limit 150,000 daltons to 6000 daltons) was obtained from Supelco (Bellefont, PA). Vydac RP C_{18} column (4.6 mD x mmL) was obtained from Separation Science, Hesperia, CA. POROS® Self Packing device, POROS®-Protein A, POROS® HQ and POROS® CM columns were obtained from PerSeptive Biosystems (Framingham, MA). Mass spectral analysis was performed on the Voyager™ BioSpectrometry Workstation with linear analyzer and a 337nm Nitrogen laser, from PerSeptive Biosystems, Inc./Vestec Mass Spectrometry Products, Framingham, MA. Peptide sequencing by Edman degradation was done on the Hewlett Packard Series II 1090 liquid Chromatograph. SDS gel electrophoresis kit was obtained from Novex Biochemicals (San Diego, CA).

3A: *Generation of Peptide Library*:

1) Natural peptide library: Twenty-three proteins were used to generate the peptide library. 60 mgs of each of the 23 proteins were mixed with 6N guanidium chloride at room temperature for half hour. 36 mM EDTA and 30 mM dithiothrietol were added to the mixture and incubated at room temperature for an additional one hour. After denaturation of proteins and reduction of disulfides, 30 mM iodoacetamide was added to the mixture which was then incubated at 37°C overnight with constant shaking. The protein mixture was dialyzed, lyophilized and divided into three batches for enzymatic digestion at 37°C for 24 hrs. One batch was treated with trypsin (protein to trypsin ratio 25:1 w/w), the second batch was treated with chymotrypsin (protein to chymotrypsin ratio 15:1 w/w) and the third batch was treated with both trypsin (1:25 w/w) and chymotrypsin (1:15 w/w). The digestion buffer used was 0.1 M ammonium bicarbonate buffer containing 0.12 mM calcium chloride (pH 8.3). After 24 hrs, the three batches were heat treated at 90°C for 30 min. to inactivate the enzymes. The efficiency of digestion was assessed by RP chromatography and SDS-polyacrylamide gel electrophoresis.

2) Anti-IgG (Fc specific) polyclonal antibodies library: Anti-IgG (Fc specific) polyclonal antibodies were obtained from rabbit, goat and sheep. 30 mg of each of the polyclonal antibodies were denatured, reduced and alkylated as described above. The mixture was treated with trypsin (1:25 enzyme:protein ratio) and the digests were pooled and lyophilized.

3) Protein A and G digests: 1 mg each of recombinant Protein A and G were denatured, reduced and alkylated as described above. The denatured proteins and 15 mgs each of native proteins A and G were treated with trypsin (1:25 enzyme:protein ratio) and chymotrypsin (1:15 enzyme:protein ratio) overnight as described above. The resulting mixture of native and denatured digests were pooled and lyophilized.

3B: *Diversity of the peptide library*:

To determine the diversity of each the peptide libraries, the sequences of all the proteins used to generate a library were obtained via the Entrez program and retrieved into the GPMAW program. The GPMAW program simulates the enzymatic digestions and generates information on the number, sequences and masses of all the possible peptides generated by such three batch digestion. This information was very valuable in predicting the extent of diversity and confirming the source, mass and sequence of the peptide(s) obtained after the screening.

3C: *Immobilization of IgG(s)*:

1 mg POROS® EP (epoxy) was suspended in 5 ml 0.1 M phosphate buffer (pH 9) containing 20 mg IgG. After the beads were well suspended, 4 ml 0.1 M phosphate buffer (pH 9) containing 2 M NA_2SO_4 were added to the mixture and it was shaken overnight at room temperature. The beads were then washed with 10 mM PBS (pH 7.5) and stored in the refrigerator before they were packed into columns.

3D: *Peptide Screening Protocol*:

1) Solution phase Peptide(s) Screening through Size Exclusion - Reversed Phase columns: 5 mg each of either mouse IgG (whole) or the Fc fragment was incubated with 20 mg of natural library and protein A and G digests, respectively. The mixture was dissolved in 1 ml of a mixture of 25 mM sodium phosphate buffer and 0.15 M NaCl (pH 7) overnight at 4°C. After incubation, several chromatographic runs through the Superdex SEC and RP columns were conducted with the protein and peptide mixture injected through the Superdex column during each run. The flow rate for the Superdex column was 0.75 ml/min with the 25 mM sodium phosphate buffer and 0.15 M NaCl (pH 7) mixture. The early eluting protein peak was collected directly onto the RP column. The remaining portion of the peak was washed off the SEC. The mouse IgG (whole) or Fc fragment and its associated peptides were then eluted from the RP column under the following conditions:

Flow rate:	1 ml/min
solvent A:	0.1% TFA/DIW
solvent B:	0.1% TFA/85% ACN/15% DIW
Gradient conditions:	0-100% B for 30 CVs.
	01.5 ml fractions were collected and lyophilized.

The following controls were run through the SEC -RP columns under similar conditions:

Control 1: Pooled peptide digests through SEC with cut off at the elution volume of mouse IgG (whole) or Fc fragment. This serves as a control for coeluting peptides.

Control 2: Mouse IgG, (whole) or the Fc fragment through columns. This serves as a control for any peptides/fragments arising due to protein degradation.

Control 3: Blank run of 0.1% TFA/DIW run through Rp column to ensure that the column was clean.

Fractions corresponding to bound peptide peaks were lyophilized and redissolved in 0.1% TFA/DIW for mass spec analysis by MALDI-TOF.

2) Solid Phase Peptide(s) Screening with immobilized target and reversed phase columns in tandem: The IgG activated POROS® was packed into 4.6 mm D x 100 mm L PEEK columns using Self Pack® assembly on BioCAD™ at 20 ml/min flow rate. 5 to 50 mgs peptide library (natural peptide library or polyclonal antibody digest) dissolved in 0.5 to 2.5 ml of 10 mM PBS (pH 7.5) was injected onto the column at flow rate of 0.5 ml/min. After wash with 5 CVs of PBS, the bound portion was eluted with 10 mM HCl and collected manually. The fraction was concentrated down to approximately 500 µl and at least 80% of it was injected onto a 4.6 mm D x 250 mm L Vydac C18 column at 1 ml/min. The RP column was equilibrated with 0.1% TFA/DIW and peptides were eluted with 15 CVs of a 0-40% acetonitrile gradient at flow rate of 1 ml/min. 1 ml fractions were collected. The fractions corresponding to the bound peptides were concentrated in Speed Vac and analyzed by MALDI-TOF and Edman sequencing. As a control for non-specific binding, the peptide library was run through a POROS® OH column (no IgG) in line with the RP column.

3E: *Analyses*:

1) Matrix Assisted Laser Desorption-Time of Flight mass spectrometry (MALDI-TOF MS): Matrix used was α-cyano-4-hydroxycinnamic acid dissolved in 1 ml of 50% acetonitrile in 0.1% TFA/deionized water. Bradeykinin and insulin were used as external standards for calibration.

2) Edman Sequencing: was done on HP 1090 sequencer with standard protocol.

3F: *Surface Design and Affinity Chromatography*:

1) Immobilization of peptides via N-terminal end: 1g POROS® AL (aldehyde) was suspended in 10mM PBS (pH 7.5) containing 100 mg sodium cyanoborohydride ($NaCNBH_3$). 5 to 20 mg of each of the peptides selected by various screening procedures was added to the resin and the mixture was shaken overnight at room temperature. 100 mg sodium borohydride ($NaBH_4$) was then added and the mixture was shaken for another 2hrs. The beads were then washed with PBS and packed into 4.6 mm D x 100 mm L column using POROS® Self Pack® device.

2) Direct synthesis of peptides on POROS®: The 19-mer peptide (TVTEKVIDASELTPAVT) selected from protein A and G digests was directly synthesized on 20 μm amine-functionalized particles by standard FMOC chemistry. About 700 mg of resin was dry packed into a 4.6 mm D x 50 mm L PEEK column equipped with 2 μm frits. The packed column was attached with appropriate adapters to a PerSeptive Biosystems 9050 plus continuous flow peptide synthesizer. Upon completion of synthesis, the resin was dried and deprotected using 95% TFA/5% triisopropylsilane for 24hrs. The final peptide-support conjugate was packed using POROS® Self Pack® column packing device at flow rate 10 ml/min onto 4.6 mm D x 50 mm L column for evaluation as affinity supports.

3G: *Analytical Methods*:

1) Purification of IgG (whole) from human serum on peptide column: 100 μl of 1:10 diluted serum was injected onto the peptide column which was equilibrated with 20 mM tris (pH 8.0) at 5 ml/min. The bound proteins were eluted with 0-1 M NaCl gradient in 15 CVs. Fractions were collected manually and concentrated by Speed Vac for subsequent analysis by SDS-polyacrylamide gel electrophoresis.

2) Purification of IgG (whole) from human serum on POROS®-Protein A column: 100 μl of 1:10 diluted serum was injected in the protein A column, which was equilibrated with 10 mM phosphate buffer containing 0.15M NaCl (pH 7.5) at a 5 ml/min flow rate. After injection and 5 CVs wash, the bound portion was eluted in a single step with 10 mM HCl. The bound fractions were collected and analyzed by SDS-Polyacrylamide gel electrophoresis.

3) Purification of IgG (whole and Fc fragment) on POROS®-peptide (TVTEKPEVIDASELPAVT) column: The 19-mer peptide column (4.6 mm D x 50 mm L) was

equilibrated with 20 CVs of equilibration buffer, 20 mM tris (pH 7). About 500 µl of 1 mg/ml of each of the following samples was injected onto the 19-mer peptide column (4.6 mm D x 50 mm L): 1) pure IgGs from either human or mouse or chicken, 2) IgA (whole molecule), 3) human serum, 4) fetal bovine serum (1:10 dilution), or 5) 500 µl of 1 mg/ml mouse Igc, Fc fragment. After injection, the column was washed with 20 CVs of a mixture of 20mM tris buffer and 0.4 M NaCl (pH 7). The remaining bound proteins were eluted with 20 CVs of 12 mM HCl. Experiments with control POROS®-NH_2 (no peptide) column were done similarly. The bound fractions were collected and analyzed by SDS-Polyacrylamide gel electrophoresis.

4) Purification of human IgG (whole) and mouse IgG (whole and Fc fragment) on POROS®-Protein A column: POROS®-Protein A column (2.1 mm D x 30mm L) was equilibrated with 20 mM tris (pH 7) for 20 CVs. About 500 µl of 1 mg/ml of each of the following samples was injected onto the POROS®-Protein A column (2.1 mm D x 30 mm L): 1) pure human IgG (whole molecule), 2) human serum (1:10 dilution), and 3) 500 µl of 1 mg/ml mouse IgG, Fc fragment. After injection, the column was washed with 20 CVs of a mixture of 20 mM tris buffer and 0.4 M NaCl, (pH 7). The remaining bound proteins were eluted with 20 CVs of 12 mM HCl. Experiments with control POROS®-OH column (no peptide) were done similarly. The bound fractions were collected and analyzed by SDS-Polyacrylamide gel electrophoresis.

5) SDS polyacrylamide gel electrophoresis: SDS-polyacrylamide gel electrophoresis of the salt and acid eluted bound fractions were run on 4-20 % or 12% (1mm x 10 cm) tris glycine precast gels under reducing conditions. Sample preparation, staining and destaining were done according to the manufacturer's recommendation.

Natural Peptide Library:

Both solution phase and solid phase peptide(s) screening of mouse IgG (whole fragment) with the natural peptide library yielded one peptide of mass 1633 daltons and amino acid sequence CAQCHTVEK. Database search revealed that this peptide is a tryptic digest of cytochrome c (one of the proteins in the library) with a heme group covalently attached to the two cysteines at amino acid positions 14 and 17 of the protein.

The CAQCHTVEK peptide with heme group was immobilized on POROS® AL (aldehyde) via the N-terminal end. This POROS®-peptide conjugate was used to separate IgG from serum at pH 8 under a 0-1M NaCl gradient. At pH 8, IgG was purified with comparable purity to that of IgG separated on POROS®-Protein A column. The capacity of the POROS®-peptide column was determined to be 10 mg/ml column volume which is comparable to the binding capacity of POROS®-Protein A column. To determine the nature of interaction, the purification profile of IgG separated on the POROS®-peptide column was compared with the IgG purified on standard ion exchange columns such as POROS®-CM and POROS® HQ. Results indicated that, under similar conditions, peptide columns exhibit predominantly ion exchange characteristics with secondary hydrophobic interactions and have a higher selectivity for IgG from serum than either of the ion exchange columns. The effect of the varying loading densities of peptide (from 10 mg/g POROS® to 100 mg/g POROS®) on specificity and capacity for IgG binding was also investigated. The specificity of the peptide for IgG binding was also investigated. The specificity of the peptide for IgG did not vary with varying ligand densities, but the nature of interaction of IgG varied. At low loading density (10 mg/g POROS®) IgG bound primarily via ionic interaction requiring elution of bound IgG with salt gradient. At higher loading density (100 mg/mg) IgG bound strongly and eluted with acid buffer. The binding capacity varied from 1-2 mg/ml column volume at lower ligand density to 30 mg/ml column volume at higher ligand densities. The heme peptide POROS® bound very weakly to HSA and only under very hydrophobic conditions (200 mM sodium sulphate, pH 7 buffer).

When the heme peptide was immobilized via the carboxyl groups of heme and the free C-terminal end, no IgG binding was seen indicating that free carboxyl groups of the peptide were very important for binding to IgG.

A simplified analog of the heme peptide (GAQGHTVEK) was synthesized and immobilized on POROS® AL via the N-terminal end. At pH 8, this GAQGHTVEK-POROS®

conjugate bound specifically, and with comparable purity, to the IgG purified from human serum on POROS®-Protein A column. The bound IgG was eluted from the GAQGHTVEK-POROS® column with 100 mM NaCl. The IgG binding capacity was determined to be 5 mg/ml column volume. Loading densities from 20 mg/g to 40 mg peptide /g POROS® were evaluated. At these ligand densities, the specificity of IgG binding was not affected, but the capacity was reduced.

Protein A and G digests:

The Fc binding domain of recombinant proteins A and G and the amino acids involved in the binding of protein A to IgG have been mapped by site-directed mutagenesis (Fahnestock, S.R., Alexander, P. Nagle, J. and Filpula, D., *J. Bacter* (1986) *167*(3):870-880). However, there has been no report of peptides isolated from these bacterial proteins that bind to IgG. By solution phase peptide(s) screening with native and denatured recombinant protein A and G digests against mouse IgG (Fc fragment) four peptides with remarkable overlapping sequences were identified. The peptide(s) were as follows: TVTEKPE, EKEPEVID, GDAPTPEKEPEASI and TVTEKPEVIDASELPAVT. The sequences of the larger peptides correlated with mass spec data. None of these peptides are typical tryptic digests, indicating that these peptides probably were selected from the native protein G digest. Database search revealed that all of these peptides were from recombinant Protein G. Further, the TVTE sequence is a part of the Fc binding domain of recombinant protein G.

The TVTEKPEV peptide was synthesized and immobilized on POROS® via the N-terminal end. This POROS®-TVTEKPEV was found to bind IgG from human serum at pH 8 with comparable specificity as the POROS®-Protein A conjugate. The bound protein was eluted with 0-1M NaCl gradient. The TVETEKPEVIDASELTPAVT peptide was synthesized directly on POROS®-NH_2 resin via the C-terminal end. This peptide bound mouse IgG, Fc fragment with low capacity but high selectivity. The bound IgG was eluted with acid buffer. This peptide was more selective for IgG than IgA. Human IgG bound more selectively to the 19-mer peptide column that IgG from rabbit, goat or mouse.

One of the important and novel features is that from proteins A & G known to bind IgG with high affinity and requiring acidic conditions to elute the bound IgG, peptides were isolated, at least one of which (TVTEK) has been shown to bind IgG, but with less affinity requiring only salt gradient for elution of the bound protein. Second, the peptide(s) of varying affinities and selectivity towards IgGs from different species have been identified. Third, it is remarkable that

two of the peptides namely, TVTEKEPEVIDASELTPAVT and TVTEKPEV are part of the Fc binding domain of recombinant protein G.

Polyclonal Antibody digests:

Polyclonal antibodies are an interesting and logical source of peptides since they have specific antigen binding sites. A synthetic antibody fragment against lysozyme has been used as a ligand in immunoaffinity chromatography. This fragment was generated by molecular modeling of lysozyme and its antibody (Welling, G.W. *et al.*, (1990) *J. Chrom., 512*:337-343). Single chain antibodies that bind with weak affinities have also been generated against many targets by phage display (Griffiths, A.D. *et al.*, (1994) *The EMBO J., 13*(14):3245-3260). To date there has been no report of selection of target specific peptide(s) isolated from polyclonal antibody digests. Tryoptic digests of denatured anti-IgG (Fc specific) polyclonal antibodies raised in rabbit, goat and sheep were run through a POROS® epoxy column immobilized with IgG. The bound peptides were eluted onto an RP column and characterized. The amino acid sequence was determined to be GAQGHTVEK. A database search revealed that the GAQGHTVEK sequence is a part of the variable region of the light chain of IgG. Note that the HTVEK motif is also found in the heme peptide of cytochrome c. The heme peptide has been shown, as above, to bind IgG. Additionally, the TVEK motif is similar to the TVTEK sequence found in the IgM heavy chain, T-cell receptor (beta chain) and also IgG binding proteins such as protein G and protein LG. Protein LG, a hybrid molecule of protein L and G, binds to intact IgGs, as well as Fc and Fab fragments and IgG light chains. The characteristics of GAQGHTVEK peptide as affinity surface for IgG binding have been discussed above. The most important and novel feature is that from a mixture of denatured antibodies, one peptide was isolated that was selective for IgG.

By choosing different libraries, peptides have been chosen that selectively bind to different portions of IgG (either the Fab or the Fc fragment). There is remarkable similarity in the sequences of some of the IgG binding peptide(s) isolated from different protein sources. None of these peptides were shown to bind IgG previously. The specificity of the peptide(s) for IgGs from various species varies depending on the orientation, activation chemistry and the density of immobilized ligand. Finally, this invention provides proof of the concept that both chromatographic peptide(s) screening technologies (solid phase and solution phase) are comparable and yield credible results.

What is claimed is:

1. An automated, fast, continuous flow, multi-dimensional method for (1) selecting based on ligand/target interaction, (2) separating, and (3) obtaining physico-chemical data characteristic of, one or more of ligands in a library of solubilized, structurally distinct candidate ligands, the method comprising the steps of:

A. passing a solution comprising said library through a first column to partition, based on a first physico-chemical property, candidate ligands, or complexes of said ligand bond to a target molecule, thereby to produce, a first exit stream; and

B. passing the exit stream, through a second column to partition, based on a second, different physico-chemical property, candidate ligands, said complexes, to generate a second exit stream comprising a subset of candidate ligands having affinity for said target molecule, wherein at least one of said first and second physico-chemical properties is the affinity of a said candidate ligand for said target molecule.

2. The method of claim 1 comprising the additional steps of sampling an exit stream containing a selected ligand after step B and inserting the sample into a mass spectrometer to determine the charge-to-mass ratio of said ligand or a fragment thereof.

3. The method of claim 1 wherein at least one of said first and second physico-chemical properties is selected from the group consisting of the affinity of a candidate ligand for:

a) a target molecule;

b) reverse phase;

c) an ion exchange surface;

d) a chelation surface;

e) a polysaccharide surface; and

f) a polynucleotide

4. The method of claim 1 wherein at least one of said first and second physico-chemical properties is selected from the group consisting of the affinity of a complex of said candidate ligand and a target molecule for:

a) a second target molecule;

b) reverse phase;

c) an ion exchange surface;

d) a chelation surface;

e) a polysaccharide surface; or

f) a polynucleotide surface.

5. The method of claim 1 wherein at least one of said first and second physico-chemical properties is selected from the group consisting of:

a) the binding constant K_b of a candidate ligand for a target molecule;

b) the on rate component of the binding constant of a candidate ligand for a target molecule; and

c) the off rate component of the binding constant of a candidate ligand for a target molecule.

6. The method of claim 1 comprising the step of conditioning said solvent in said exit stream prior to partioning in said second column.

7. The method of claim 1 wherein the first column comprises an affinity column comprising immobilized first target molecules.

8. The method of claim 7 wherein only candidate ligands which fail to bind to said first target molecules are passed through the second column thereby to eliminate candidate ligands which bind to the first target molecule from subsequent screening.

9. The method of claim 8 wherein candidate ligands which bind to said first target molecules are desorbed from said first column and passed through the second column thereby to eliminate candidate ligands which fail to bind to the first target molecule from subsequent screening.

10. The method of claim 8 wherein said second column comprises an affinity column comprising immobilized second target molecules.

11. The method of Claim 9 wherein said second column comprises an affinity column comprising immobilized second target molecules.

12. The method of claim 1 wherein said first column comprises a size exclusion column and, prior to step A, said library is mixed with a target molecule to form candidate ligand/target molecule complexes.

13. The method of claim 1 wherein said second column is a reverse phase column which captures ligands in the exit stream of said first column.

14. A method of detecting in a heterogeneous sample comprising a multiplicity of candidate ligand species a ligand having a high affinity for a target molecule, the method comprising the steps of:

a) digesting biopolymers to produce oligomeric fragments thereof comprising said ligand species;

b) combining with said ligand species produced in step a) with a target molecule, under conditions which will allow candidate ligands, if present, to bind to the target molecule to form a complex; and

c) separating candidate ligands from said complex.

15. The method of claim 14 wherein one or more biopolymers comprise a known binder to said target molecule, or a derivatized or modified form of said known binder.

16. The method of claim 14 wherein said step b) comprises passing the candidate ligand species over a target molecule immobilized on an affinity column, thereby to immobilize on said column a complex comprising said target molecules and candidate ligands and thereafter eluting ligands from said column.

17. The method of claim 16 comprising the additional steps of: eluting ligands into an accumulator to capture a ligand; and eluting said captured ligand from said accumulator.

18. The method of claim 17 wherein isolated ligand is identified by a process comprising determing the mass-to-charge ratio of ions generated therefrom.

19. The method of claim 14 wherein step a) is effected by, immobilizing one or more digestive enzymes on a column and passing one or more biopolymers to be digested through the column to obtain the said oligomeric fragments.

20. The method of claim 14 wherein step b) comprises mixing the candidate ligand species with said target molecule to form soluble complexes thereof and separating complexes from uncomplexed ligand species on a size exclusion chromatography column.

21. A method of detecting the presence of a ligand having a preselected affinity K for a preselected target molecule in a sample of heterogeneous ligands dissolved in a solvent, the method comprising:

a) loading a column with a known concentration T of target molecules;

b) passing a sample through said column to bind ligands in the sample thereto;

c) thereafter passing through the column a series (n) of column volumes of solvent, wherein n is a number of column volumes between 1 and 10,000;

d) passing a subset KT of the column volumes exiting the column of step c) through a ligand accumulator to immobilize thereon ligands having said preselected affinity K wherein the subset of column volumes kp~KT, and,

e) eluting from the accumulator of step d) said ligands having said affinity K.

22. The method of claim 21 wherein, during step b), the velocity of the sample passing through the column is selected to modulate the on-rate component of the affinity constant of the ligand sought to be detected, wherein increasing the velocity of the sample through the column results in the binding in step b) and elution in step e) of ligands having a larger on-rate.

23. The method of claim 21 wherein, during step c), the velocity of solvent passing through the column is selected to modulate the off-rate component of the affinity constant of the ligand sought to be detected, wherein increasing the velocity of the solvent through the column results in the elution in step e) of ligands having a higher off-rate.

24. The method of claim 21 comprising the step of passing the eluate of step (d) through an interface to a mass spectrometer.

25. The method of claim 21 further comprising the step of independently optimizing the flow rate through the columns of step a) and b), and independently optimizing the flow rate of the eluate to a mass spectrometer for determination of the mass-to-change ratio of a ligand in said eluate.

26. A method for separating mixed ligand species dissolved in a solvent into separate fractions of ligands, each fraction being characterized by a different affinity or range of affinities for a preselected target molecule, the method comprising:

a) passing the mixed ligand species through a column having immobilized thereon target molecules to bind ligands to the column;

b) passing through the column a series of column volumes of solvent;

c) passing at least two subsets of the column volumes of solvent exiting the column of step b) through a ligand accumulator to immobilize thereon ligands characterized by separate ranges of affinity constants; and

d) eluting from the accumulator of step c) said at least two fractions containing ligands characterized by different ranges of affinities.

27. The method of claim 26 further comprising diverting consecutive subsets of the column volumes of solvent exiting the column of step b) to different accumulator columns.

28. A method of assay for rapidly detecting the presence, absence or concentration of an analyte in a sample, said analyte having a known affinity constant K in a preselected solvent for a binding molecule, the method comprising the steps of:

a) providing a matrix comprising a column having loaded therein immobilized binding molecule having the affinity K for the analyte;

b) passing a sample through the matrix to bind analyte to said binding molecule;

c) passing through the matrix a series (n) of column volumes of the solvent, wherein n is a number of column volumes between 1 and 10,000;

d) passing a subset kp of the column volumes exiting the column of step c), which subset contains said analyte, if present, through an analyte accumulator to immobilize thereon analyte having said preselected affinity K, wherein the subset of column volumes kp~KT, wherein T is the concentration of binding molecules in said matrix;

e) eluting analyte present in the accumulator of step d) to produce an eluate; and

f) detecting the absence, presence or concentration of analyte in said eluate.

29. A method of detecting in a heterogeneous sample comprising a multiplicity of ligand species the presence of a ligand having a desired high affinity K for a preselected target molecule when said ligand and said target molecule are present together in preselected solvent conditions, the method comprising:

a) immobilizing a target molecule onto a column;

b) passing the sample through said column under conditions to promote binding of ligands in the sample to the target molecules;

c) thereafter passing through the column a series of column volumes of solvent defining said solvent conditions;

d) passing a subset kp of the column volumes exiting the column of step c) through a ligand accumulator to immobilize thereon ligands having said desired high affinity, and

e) eluting said ligands from the accumulator of step d).

30. The method of claim 29 wherein a ligand eluted in step e) is characterized by a said high affinity K for said target molecule equal approximately to kp/T where T is the concentration of target molecules in said column.

31. A method of detecting, in a heterogeneous sample comprising multiple ligand species at least some of which bind a preselected target molecule with an affinity of at least about 10^4 M^{-1}, the presence of a ligand having a high on-rate, Ko, when said ligand and said target molecule are present together in preselected solvent conditions, the method comprising:

a) immobilizing a target molecule onto a column;

b) providing said heterogeneous sample in a solvent defining said preselected solvent conditions;

c) passing the sample of step b) through said column at a high linear fluid velocity so as to minimize residence time of ligands of said sample in said column thereby to bind selectively high on-rate ligands to said target molecules in preference to other ligands in the sample;

d) thereafter eluting said column to produce an output and identifying said high on-rate ligands.

32. The method of claim 31 comprising the additional step of passing the output of step d) through a ligand accumulator and eluting the accumulator to produce an output rich in said high on-rate ligand.

33. A method for the selective screening of a library of heterogeneous ligands to detect a desired ligand characterized by at least two different preselected binding characteristics to first and second target molecules, said method comprising the steps of:

a) combining a solution of heterogeneous ligands with a first target molecule under conditions such that candidate ligands bind to said first target molecule thereby to form candidate ligand/first target molecule complex;

b) passing the complex of step a), or candidate ligands separated from said complex, through an affinity column containing immobilized second target molecule; and

c) collecting from said column those ligands having the desired binding characteristics.

34. The method of claim 33 wherein one of step a) and step b) is performed by introducing the library to a column having the first target molecule immobilized thereon.

35. The method of claim 33 wherein one of step a) and step b) is performed by introducing the complex and unbound components to a size exclusion column and collecting the complex therefrom.

36. The method of claim 33 further comprising passing solution exiting the column of step b) through an accumulator to immobilize thereon ligands having the desired binding characteristics.

37. An instrument for isolating selected ligands

which have an affinity for and display preferential binding to a preselected target molecule, in amounts sufficient to permit physico-chemical structural characterization thereof,

the instrument comprising structure defining a variable flow path controllable by the user for passing solutions through various paths thereof and, disposed along said flow path:

an inlet for receiving a mixed solution containing at least a multiplicity of different candidate ligands;

a first column, in fluid communication with said inlet, for partitioning candidate ligands, or complexes thereof with a target molecule, on the basis of the varying affinity of said candidate ligands for a target molecule;

a second column, for receiving an output from said first column, for further partitioning ligand species in said output on the basis of interaction with a different target molecule, one of said target molecules comprising said selected target molecule; and

a valve interposed between an outlet of said first column and an inlet of said second column for directing a selected portion of the output of said first column to said second column.

38. The apparatus of claim 37 further comprising at least one additional column for partitioning ligands, or complexes of ligands with a target molecule, passing therethrough, on the basis of interaction with still another different target molecule.

39. The apparatus of claim 38 wherein said at least one additional column is interposed between said first and second columns and the output from said first column passes through said additional column before entering said second column.

40. The apparatus of claim 38 wherein said at least one additional column is disposed after said second column and is adapted to receive at least a portion of an output from said second column.

41. The apparatus of claim 37 further comprising a ligand accumulator connected through a valve to an output of said second column or a said additional column for capturing a subset of ligands characterized by said affinity for and preferential binding to said preselected target molecule.

42. The apparatus of claim 41 wherein said accumulator is a reverse phase chromatography column, which permits chromatographic separation of accumulated ligands.

43. The apparatus of claim 37 further comprising an instrument for determining a physico-chemical structure aspect of a selected ligand exiting a said column or accumulator.

44. The apparatus of claim 43 wherein said means is a mass spectrometer.

45. The apparatus of claim 37 disposed between consecutive columns for conditioning the solvent characteristics of an output stream of an upstream column for partitioning within a downstream column.

46. The apparatus of claim 37 for isolating selected ligands which will bind to target molecules from a first biological source but not to structurally related target molecules from a second biological source, wherein said first unit is an affinity column comprising immobilized target molecules from said second source, said second unit is an affinity column comprising immobilized target molecules from said first source, and said valve directs ligands which do not bind to said first for capture in said second unit.

47. An integrated multi-dimensional system for isolating and for obtaining physico-chemical data characteristic of ligands having an affinity for a preselected target molecule, said system comprising,

structure defining a variable flow path controllable by the user for passing solutions through various paths thereof and, disposed along said flow path:

an affinity column comprising immobilized target molecules;

an accumulator in fluid communication with an output from said affinity column; and

a mass spectrometer for determining the charge-to-mass ratio of ligands eluted from said accumulator.

48. The system of claim 47 further comprising an interface between the accumulator and the mass spectrometer, the interface comprising:

a) a sampler having a predetermined sample volume switchable alternatively to be in fluid communication with an eluate stream of the accumulator and an input of the mass spectrometer; and.

b) a sampler controller for controlling the sampler to first extract a sample from said eluate stream thereby to capture a sample of eluate and thereafter to insert the captured sample into the input of the mass spectrometer.

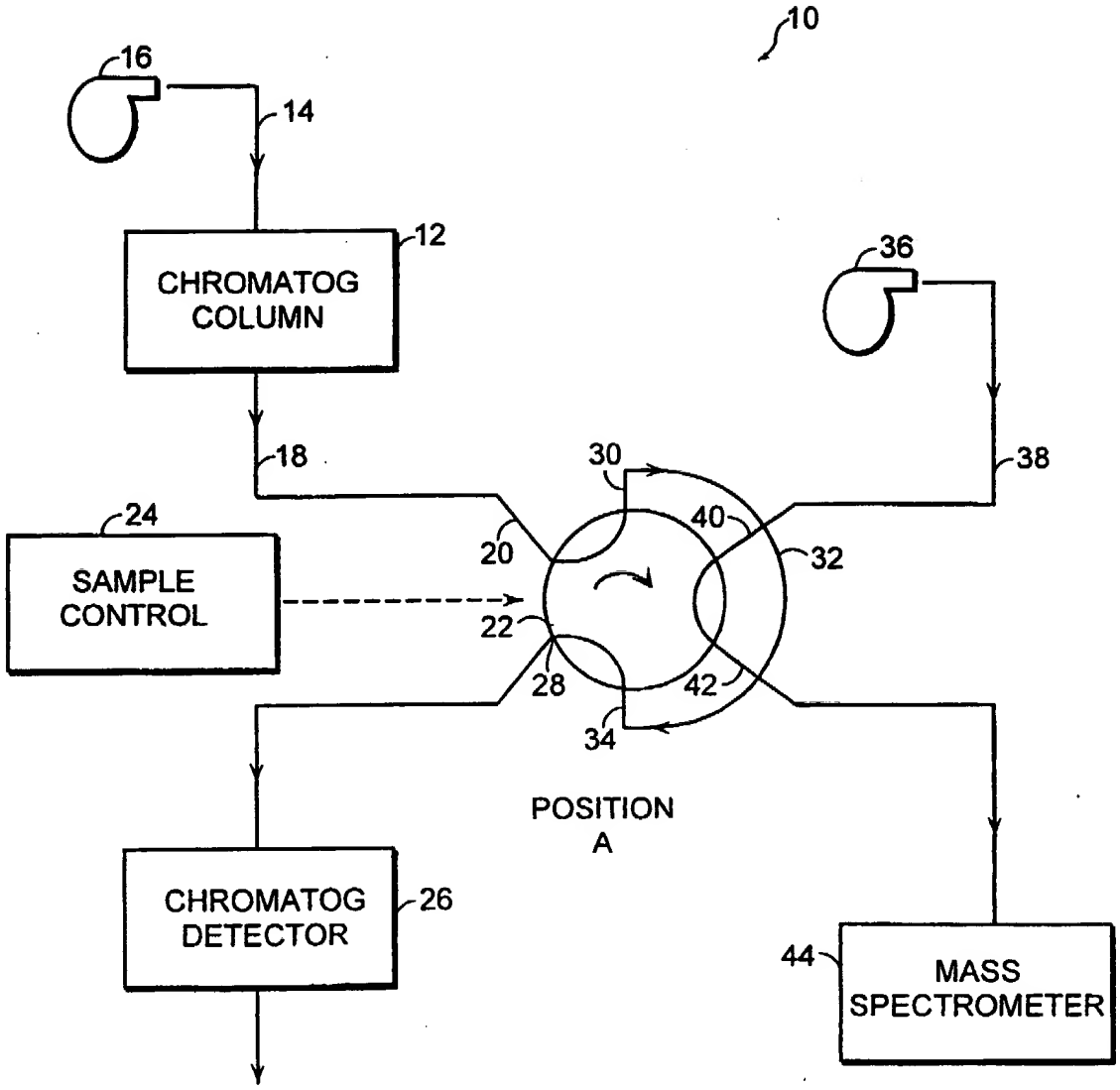
10
16
14
CHROMATOG COLUMN
12
36
18
30
38
24
40
20
32
SAMPLE CONTROL
22
28
42
34
POSITION A
CHROMATOG DETECTOR
26
44
MASS SPECTROMETER

FIG. 1

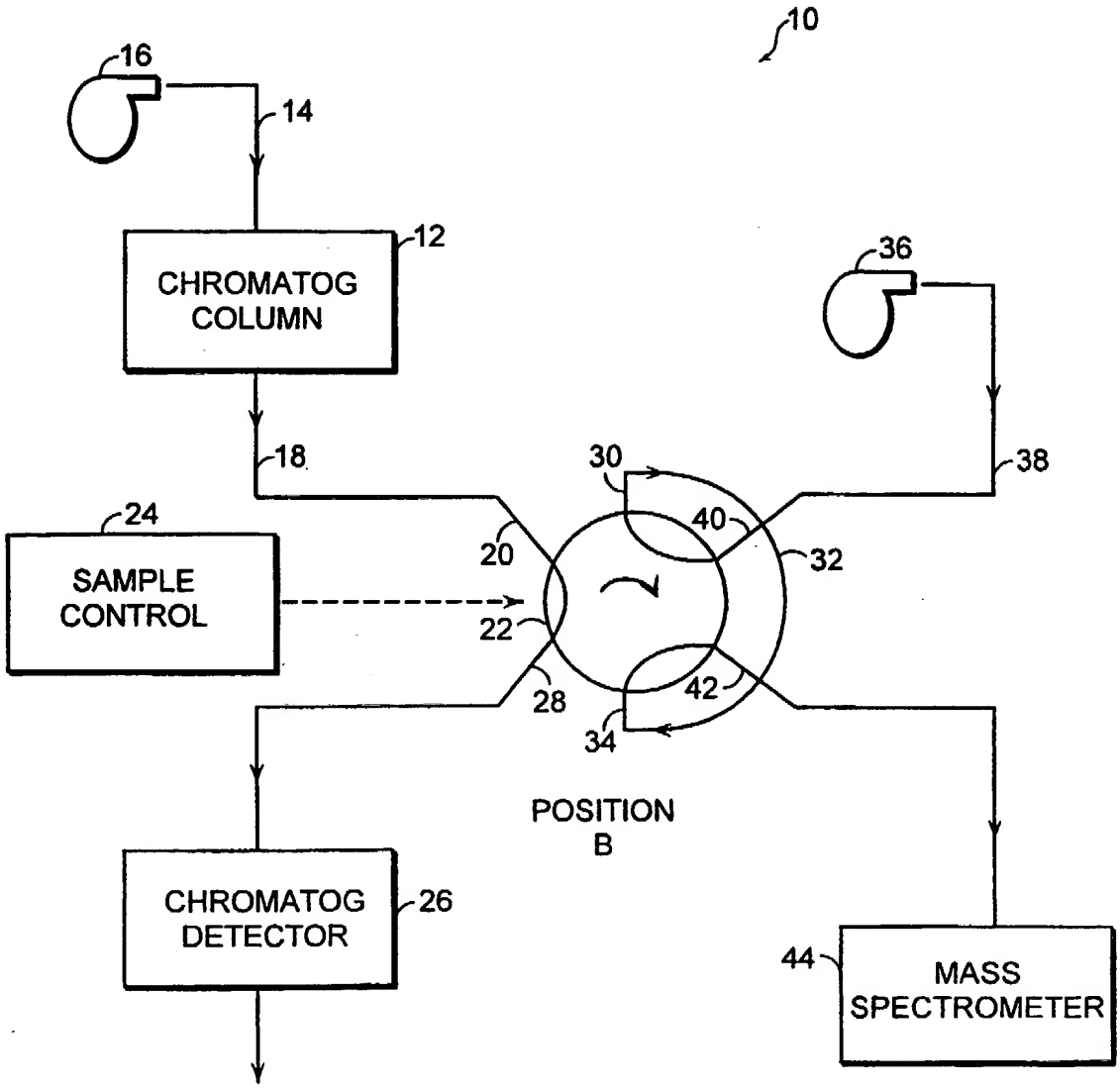
10
16
14
CHROMATOG COLUMN
12
36
18
30
38
24
40
SAMPLE CONTROL
20
32
22
28
42
34
POSITION B
CHROMATOG DETECTOR
26
44
MASS SPECTROMETER

FIG. 2

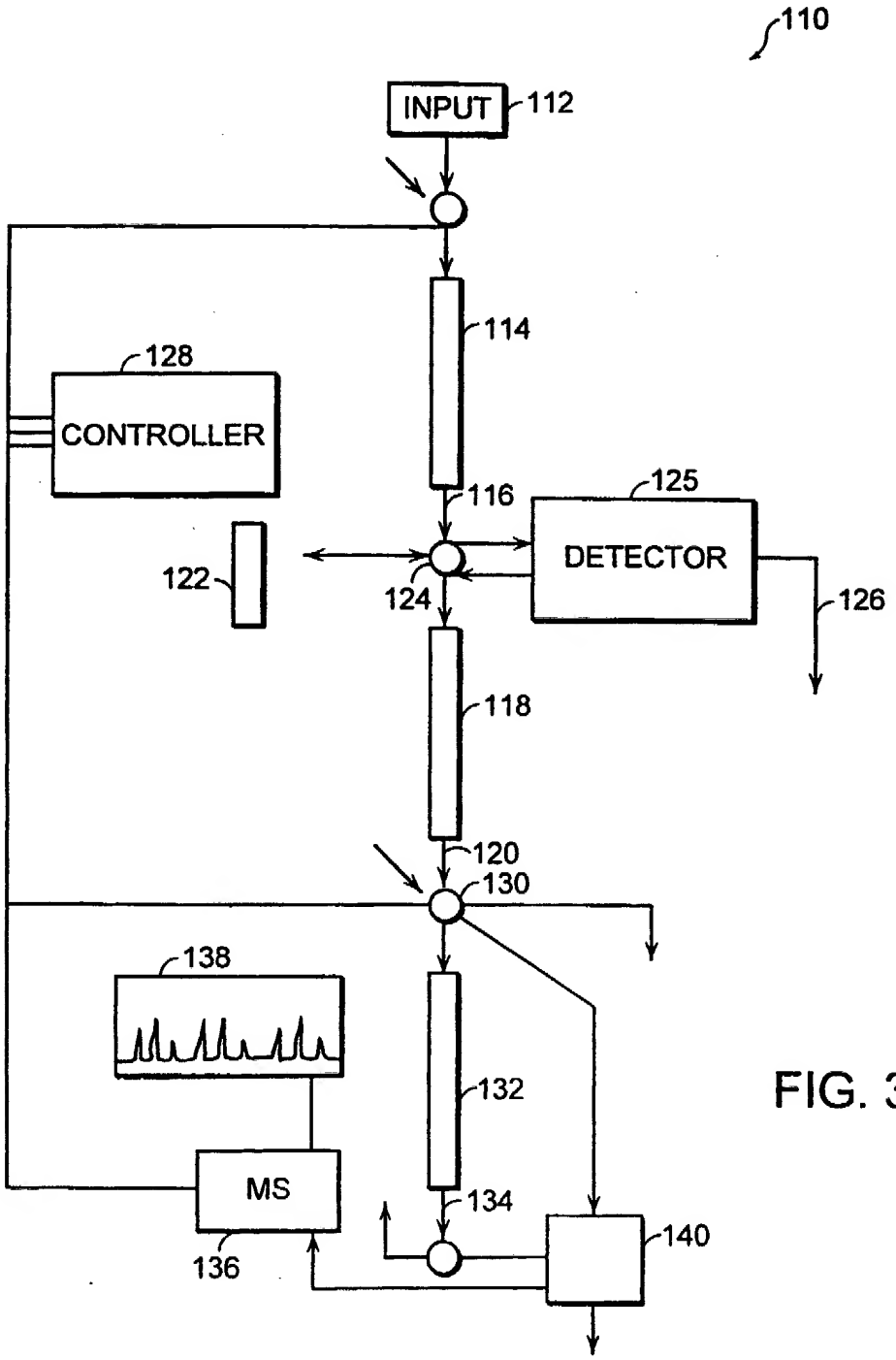
110
INPUT
112
114
128
CONTROLLER
116
125
DETECTOR
122
124
126
118
120
130
138
132
MS
134
140
136

FIG. 3

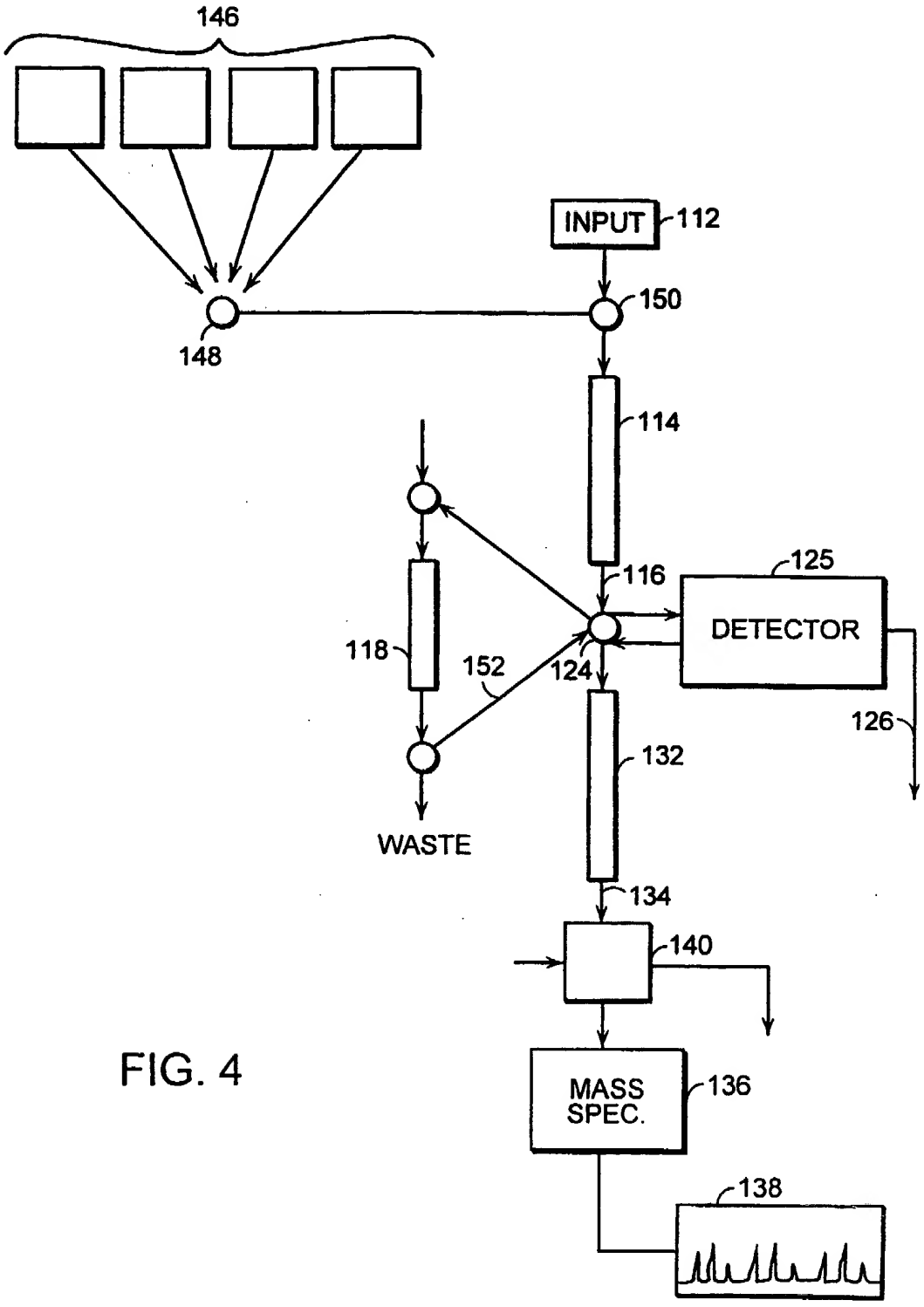
146
INPUT
112
150
148
114
125
116
DETECTOR
118
152
124
126
132
WASTE
134
140
MASS SPEC.
136
138

FIG. 4

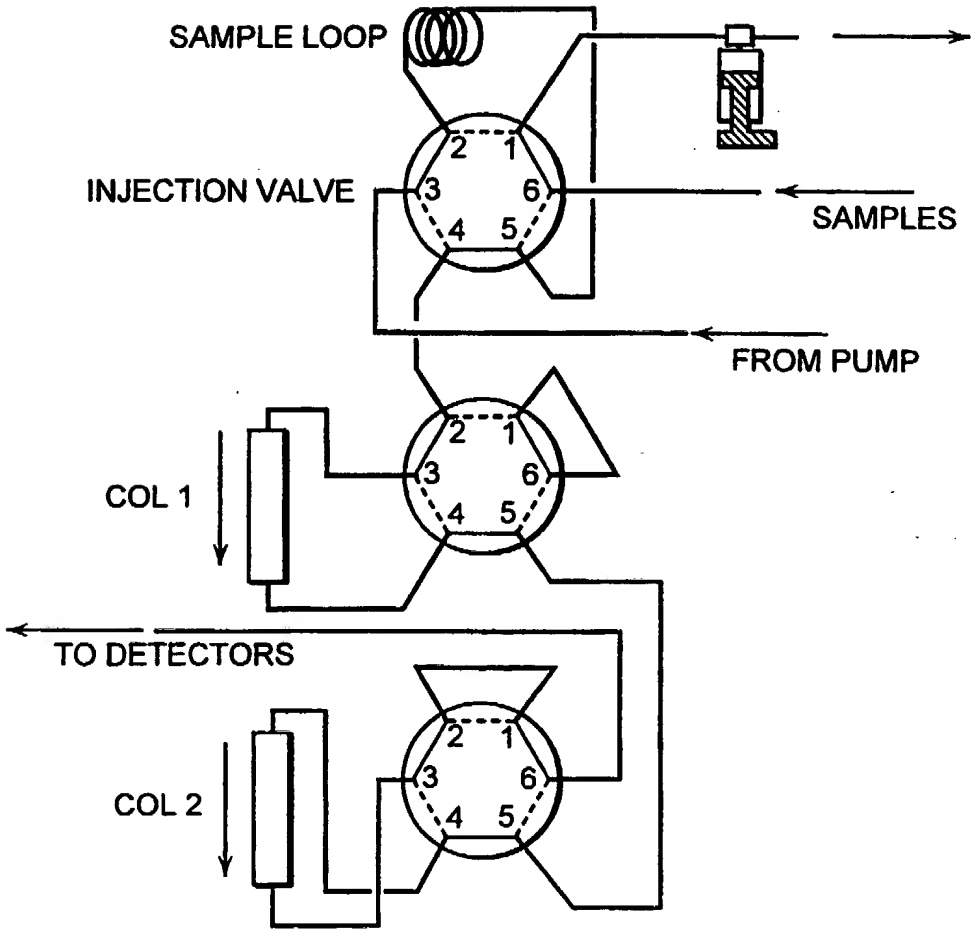
SAMPLE LOOP
INJECTION VALVE
SAMPLES
FROM PUMP
COL 1
TO DETECTORS
COL 2
1
2
3
4
5
6

FIG. 5

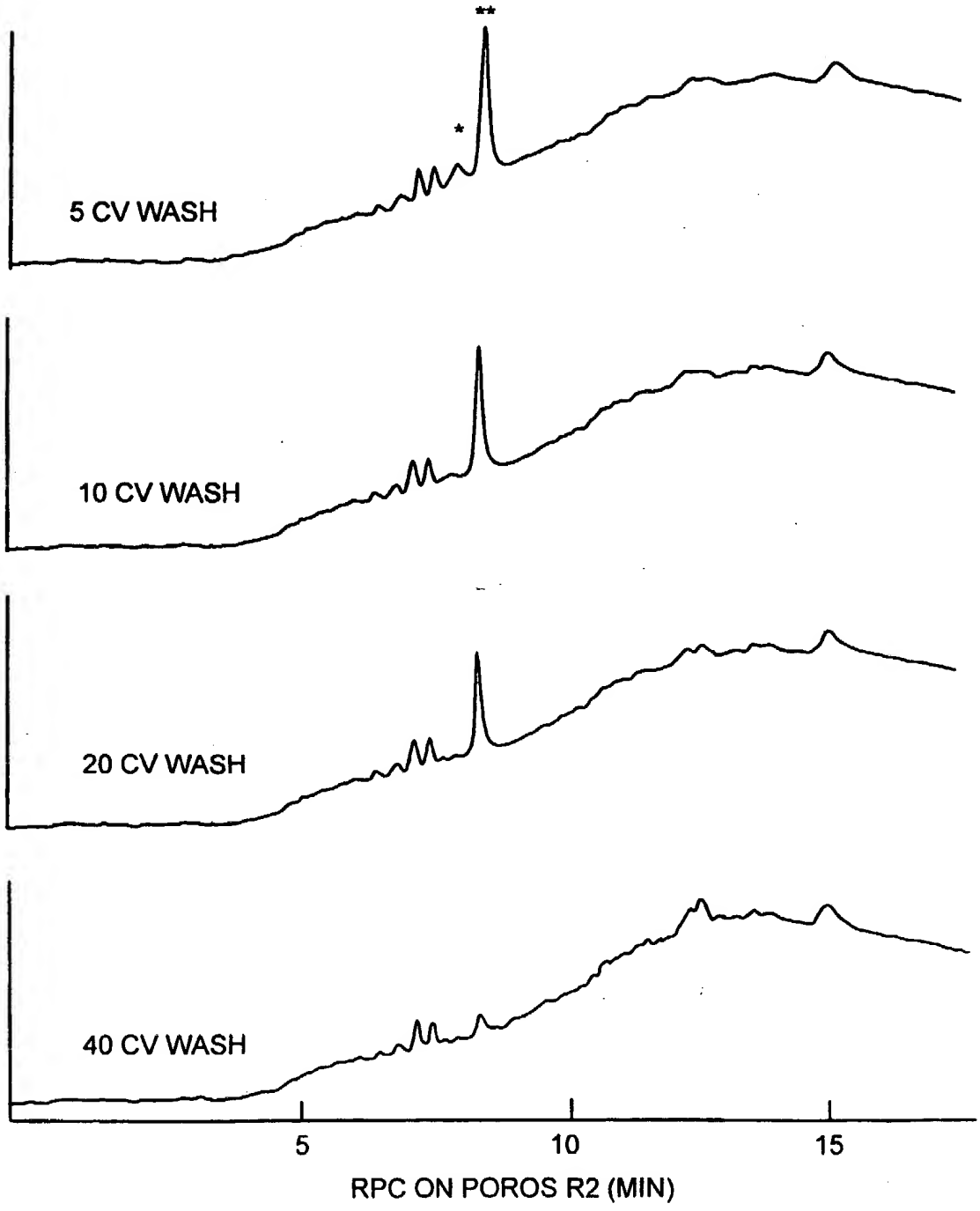
TARGET-BASED SCREENING OF HUMAN rHsp70
**
*
5 CV WASH
10 CV WASH
20 CV WASH
40 CV WASH
5
10
15
RPC ON POROS R2 (MIN)

FIG. 6

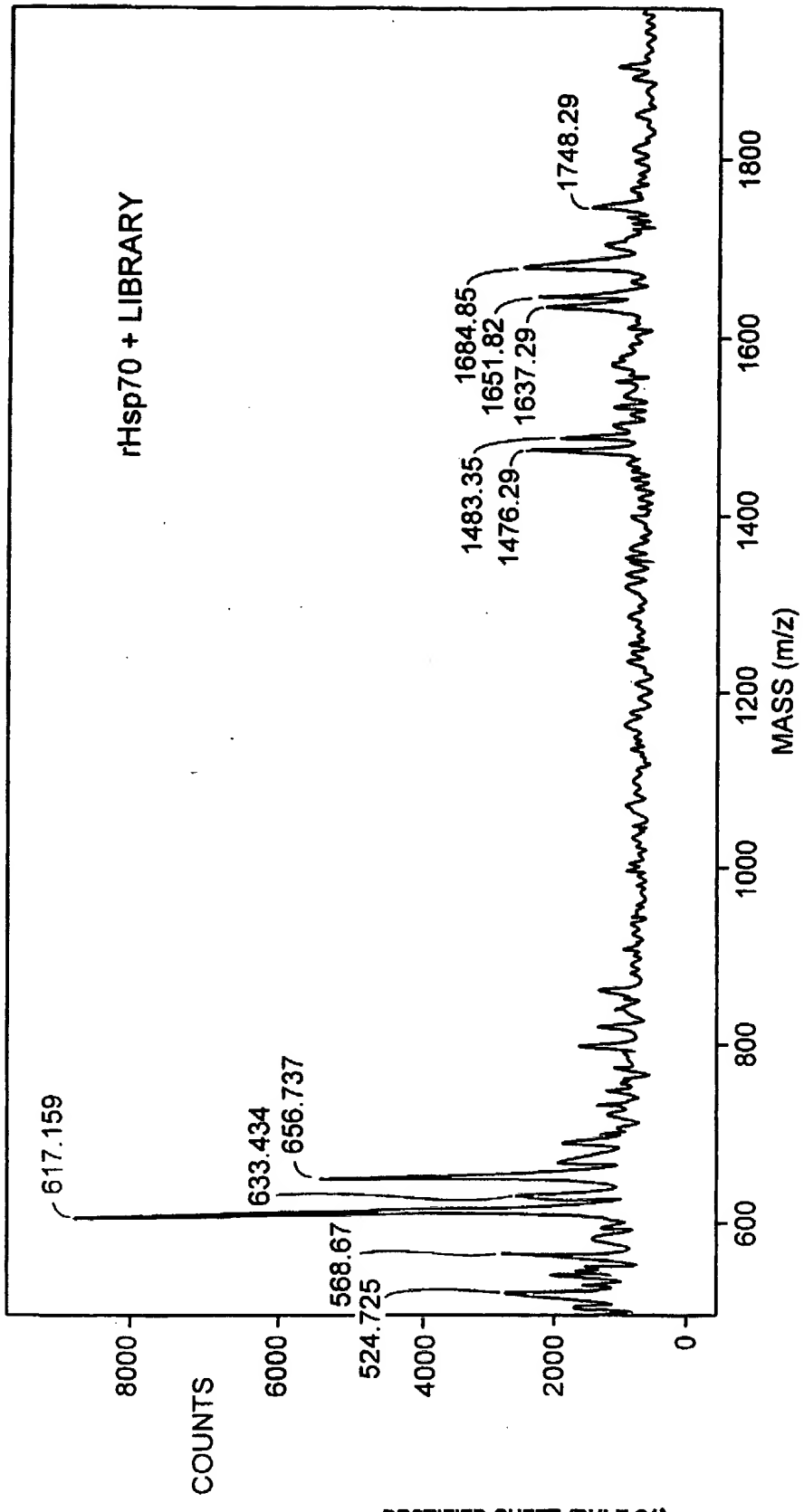
rHsp70 + LIBRARY
617.159
633.434
656.737
568.67
524.725
1483.35
1476.29
1684.85
1651.82
1637.29
1748.29
COUNTS
8000
6000
4000
2000
0
600
800
1000
1200
1400
1600
1800
MASS (m/z)

FIG. 7A

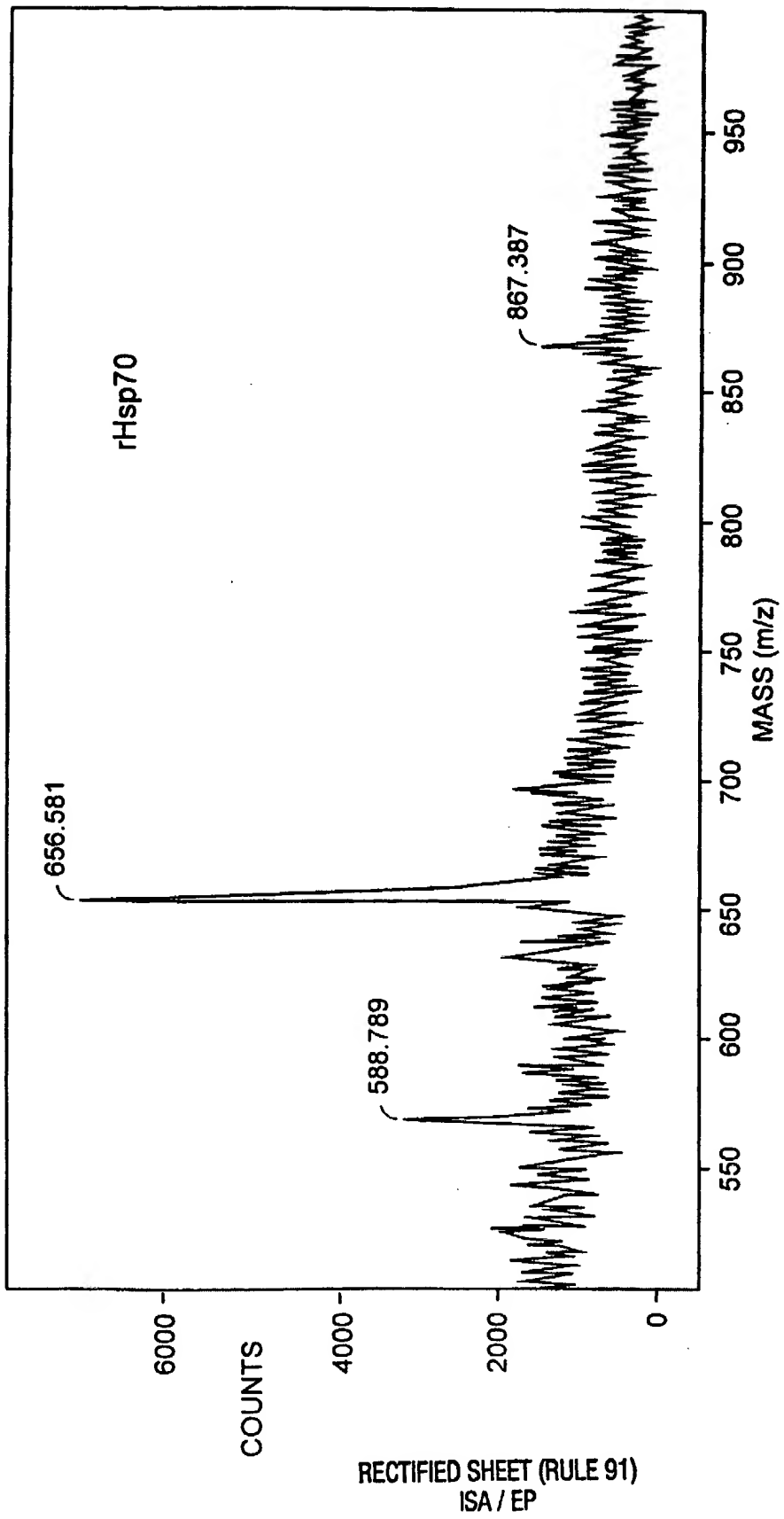
rHsp70
656.581
588.789
867.387
COUNTS
6000
4000
2000
0
550
600
650
700
750
800
850
900
950
MASS (m/z)

FIG. 7B

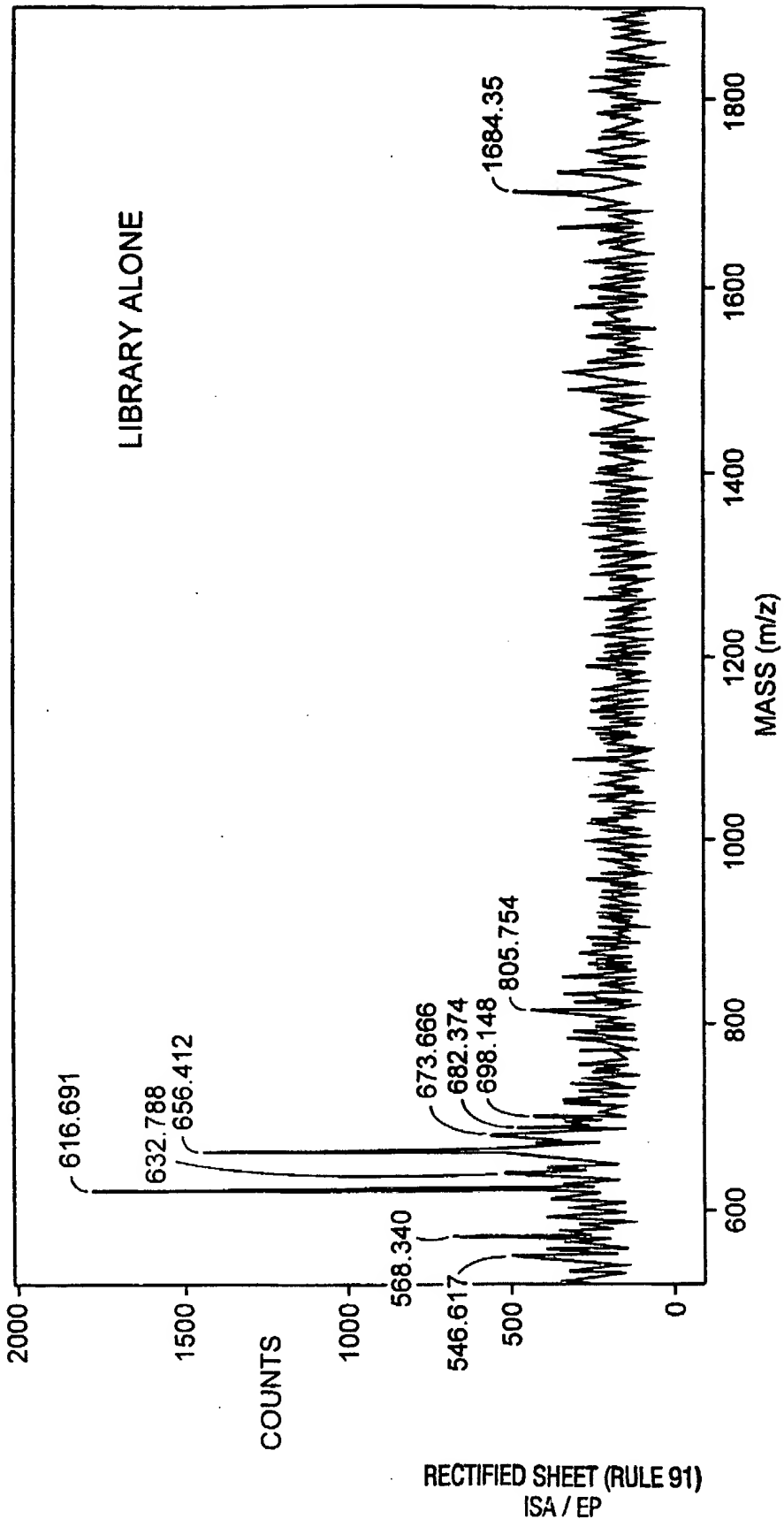
LIBRARY ALONE
COUNTS
MASS (m/z)
2000
1500
1000
500
0
600
800
1000
1200
1400
1600
1800
616.691
632.788
656.412
673.666
682.374
698.148
805.754
1684.35
568.340
546.617

FIG. 7C

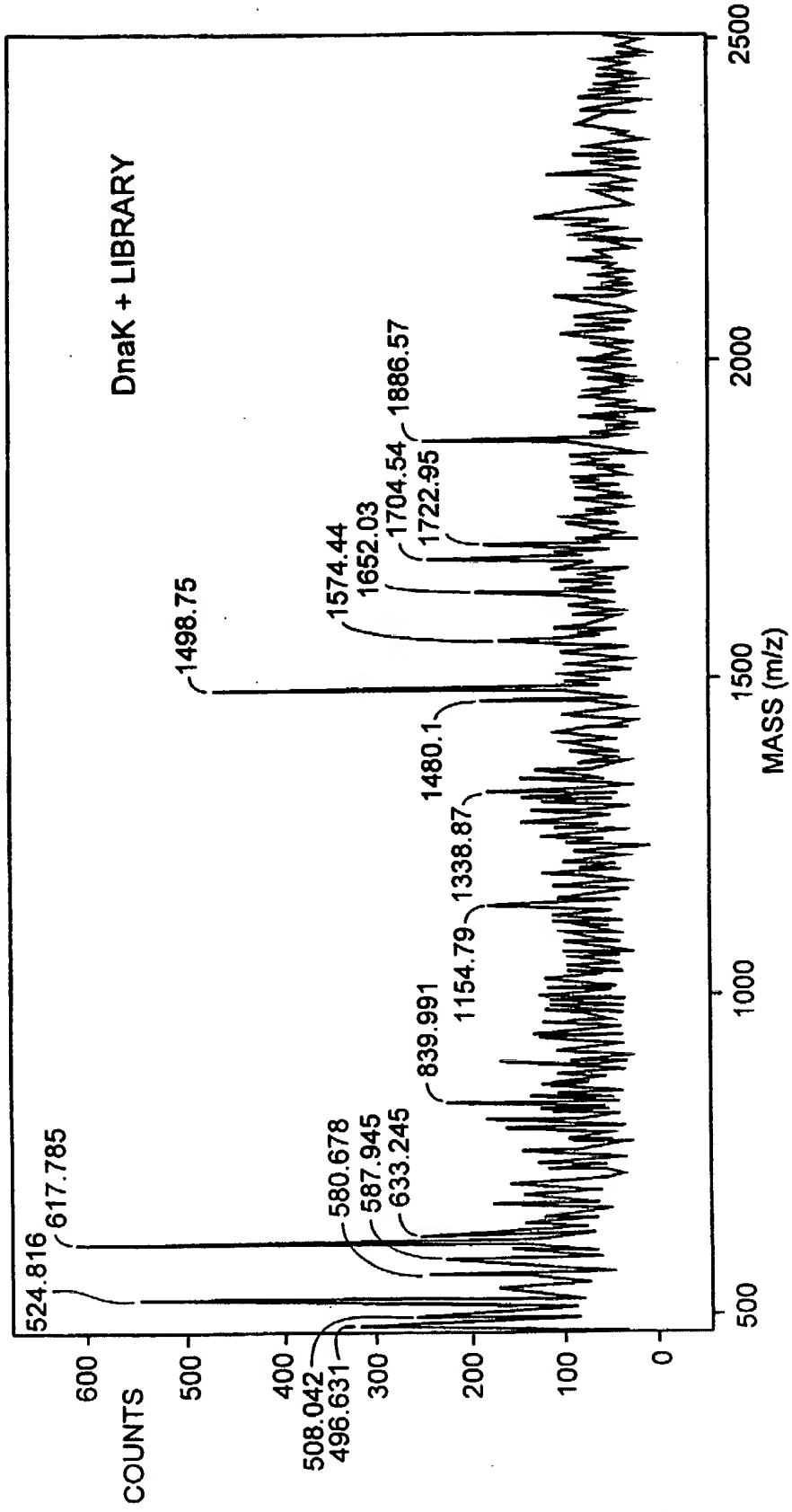
DnaK + LIBRARY
COUNTS
MASS (m/z)
0
100
200
300
400
500
600
500
1000
1500
2000
2500
524.816
617.785
580.678
587.945
633.245
839.991
1154.79
1338.87
1480.1
1498.75
1574.44
1652.03
1704.54
1722.95
1886.57
508.042
496.631

FIG. 7D

IDENTIFICATION OF PEPTIDE SEQUENCES
BINDING TO CONCANAVALIN A IN A
SUGAR-SPECIFIC MANNER*

RANKING AMINO ACIDS FROM GREATEST TO LEAST ENRICHMENT OVER CONTROL

NO INCUBATION ON-COLUMN						30MIN INCUBATION ON-COLUMN							
CYCLE #						CYCLE #							
1	H	R	Q	N		1	W	H	Q	R	I	V	N
2	H	N	Q			2	W	V	T	Q	I	P	
3	R	H/D/Q/N				3	W	V	T	Q	D	E	
4	S	R	W	Y		4	W	S	Q	V	E	N	
5	Y	R	Q			5	V	T	W	Q	E/D/G	N	R

*TABLE SHOWING ENRICHMENT OF AMINO ACIDS AT EACH CYCLE IN THE ABSENCE AND PRESENSE OF SUGAR

FIG. 8

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Jim Wells et al.	Docket No.:	39750-0002DV1
Serial No.:	09/981,547	Group Art Unit:	1639
Filing Date:	October 17, 2001	Examiner:	Epperson, Jon D.

For: **METHODS FOR RAPIDLY IDENTIFYING SMALL ORGANIC MOLECULE LIGANDS FOR BINDING TO BIOLOGICAL TARGET MOLECULES**

Commissioner of Patents &Trademarks
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF GARY SIUZDAK, Ph.D.

Sir:

I, GARY SIUZDAK, Ph.D. declare that:

1. I am currently the Senior Director at the Center for Mass Spectrometry and Associate Professor of Molecular Biology at The Scripps Research Institute. My field of expertise includes the use of mass spectrometry in biological applications. My degrees include a B.S. in Chemistry and a B. A. in Mathematics from Rhode Island College (1985) and a Ph.D. in Physical Chemistry from Dartmouth College (1990).

2. Among my many publications in the field of mass spectrometry include the book MASS SPECTROMETRY FOR BIOTECHNOLOGY, New York, Academic Press (1996). As I understand it, an Examiner in the Patent and Trademark Office has cited Chapter 6 (titled Specific Applications), pages 119-126, in the examination of a patent application. The claimed invention relates to a screening method where novel ligands are identified by subjecting a mixture of a target protein and a plurality of potential ligands that are each capable of forming a covalent bond with the protein to mass spectrometry analysis, and detecting the most abundant covalently bound protein-ligand conjugate that is formed, and identifying the ligand present in the conjugate.

3. As I understand it, the Examiner finds similarity between the claimed invention and the specific applications described in pages 119-126 of my book, particularly the analysis of the mechanism of catalytic antibodies and enzymes where mass spectrometry was used to study the covalently bound substrate-antibody/enzyme intermediates of the respective reactions. Because the cited portion ends with a statement that "[e]lectrospray ionization mass spectrometry has also demonstrated its potential...for observing covalent protein-bound intermediates in an antibody-catalyzed reaction," the Examiner appears to have concluded that this statement would have provided a person skilled in the art with motivation to use electrospray ionization mass spectrometry, or mass spectrometry in general, to identify novel ligands of proteins by detecting covalently bound ligand-protein conjugates in a similar manner.

4. I respectfully disagree with the Examiner that the cited statement would have motivated a person skilled in the art to identify a novel ligand by the mass spectrometry detection of a covalently bound protein-ligand conjugate in a mixture.

5. Studies of enzymatic mechanisms involve the detailed characterization of a single reaction where the participants, namely an enzyme and its substrate, are known. As a result, the important aspect of these studies is not determination of the identity of the binding partner of the enzyme (which is known) but to detect the non-covalent binding of the substrate to the enzyme, the formation of the covalent enzyme-substrate intermediate, and finally the dissociation of the product and the enzyme.

6. While electrospray ionization mass spectrometry is well suited to study enzymatic mechanisms where all of the participants are known, its use to analyze mixtures of unknown components is limited. Because heterogeneous compounds can produce complicated spectra that can be difficult or impossible to interpret, samples for electrospray ionization mass spectrometry usually have to be of very high purity. Another obstacle for the use of this technique is that heterogeneous mixtures tend to reduce the sensitivity of electrospray ionization mass spectrometry. Many of these obstacles are shared by other techniques of mass spectrometry.

7. Consequently, I do not believe that a person skilled in the art would have assumed that the mass spectrometry techniques to study enzymatic mechanisms would have been applicable to identify novel ligands by the mass spectrometry analysis of a mixture of unknown chemical entities, detecting a covalently bound protein-ligand conjugate from among the chemical entities present in the mixture, and determining the identity of the ligand present in the conjugate detected.

I further declare that all statements made herein of my own knowledge are true; and that these statements were made with the knowledge that willful false statements are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: February 15, 2005

By: Gary Siuzdak

Gary Siuzdak, Ph.D.